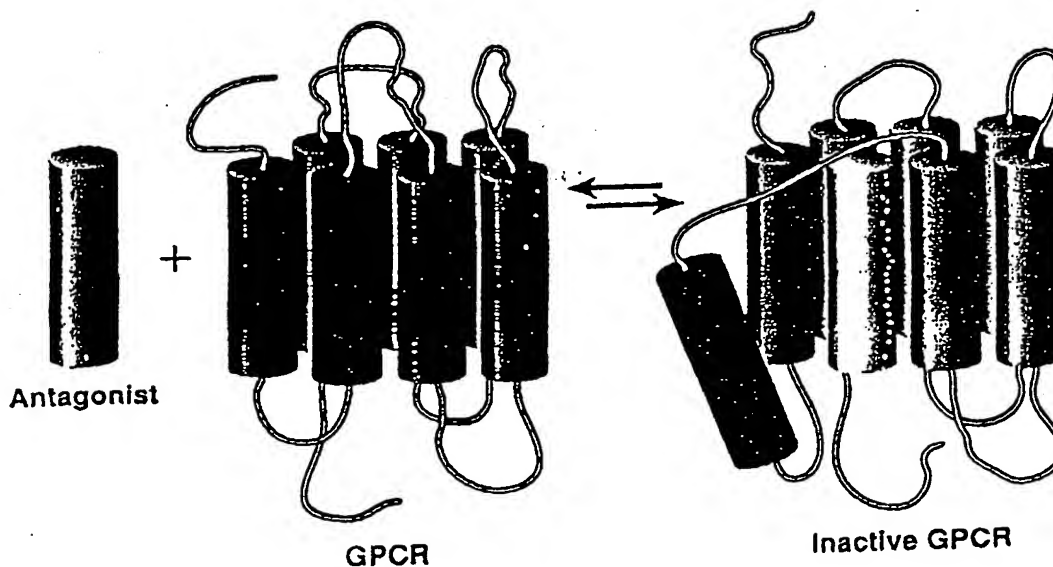




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<p>(21) International Application Number: <b>PCT/US99/04438</b></p> <p>(22) International Filing Date: <b>26 February 1999 (26.02.99)</b></p> <p>(30) Priority Data: <b>60/076,105</b> <b>27 February 1998 (27.02.98)</b> <b>US</b></p> <p>(71) Applicant (for all designated States except US): <b>THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Bethesda, MD 20892 (US).</b></p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): <b>TARASOVA, Nadya I. [RU/US]; 103 Pipe Meadow Way, Frederick, MD 21702 (US). MICHEJDA, Christopher, J. [US/US]; 13814 Hidden Glen Lane, Potomac, MD 20878 (US).</b></p> <p>(74) Agents: <b>WEBER, Kenneth, A. et al.; Townsend and Townsend and Crew LLP, 8th floor, Two Embarcadero Center, San Francisco, CA 94111-3834 (US).</b></p>	<p>(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</b></p> <p><b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	

(54) Title: **G PROTEIN-COUPLED RECEPTOR ANTAGONISTS**

## (57) Abstract

G-protein coupled receptors (GPCR) generally contain seven transmembrane helices. The present invention provides synthetic peptides derived from these transmembrane helices. The peptides inhibit GPCR function by disrupting GPCR structure. In certain embodiments, charged residues are added at one terminus to promote correct orientation of the peptide in the membrane.

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## G Protein-Coupled Receptor Antagonists

### CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/076,105 filed 2/27/98, the disclosure of which is incorporated by reference.

10

### FIELD OF THE INVENTION

15

The present invention relates to modulating, especially inhibiting, biological activities of G protein coupled receptors (GPCRs) by exposing GPCRs to molecules which interfere with correct receptor assembly. In particular, the invention relates to synthetic, isolated and/or recombinant peptides, fragments and/or consensus peptides of the transmembrane domain of GPCRs that inhibit GPCR-mediated signal transduction.

### BACKGROUND OF THE INVENTION

20

Many physiologically important events are mediated by the binding of guanine nucleotide-binding regulatory proteins (G proteins) to G protein-coupled receptors (GPCRs). These events include vasodilation, stimulation or decrease in heart rate, bronchodilation, stimulation of endocrine secretions and enhancement of gut peristalsis, development, mitogenesis, cell proliferation and oncogenesis.

25

30

G proteins are a diverse superfamily of guanine nucleotide-binding proteins that play a central role in signal transduction and regulation of cellular metabolism. They are generally comprised of three subunits: a guanyl-nucleotide binding alpha subunit; a beta subunit; and a gamma subunit. (For a review, see Conklin et al. *Cell* 73, 631-641, (1993)). G proteins commonly cycle between two forms, depending on whether GDP or GTP is bound to the alpha subunit. When GDP is bound, the G protein exists as a heterotrimer, the G alpha-beta-gamma complex. When an alpha-beta-gamma complex operatively associates with a ligand-activated GPCR in a cell membrane, the rate of exchange of GTP for bound GDP is increased and the G alpha subunit dissociates from the G beta-gamma complex. The

free G alpha subunit and G beta-gamma complex are capable of transmitting a signal to downstream elements of a variety of signal transduction pathways, for example by binding to and activating adenylyl cyclase. This fundamental scheme of events forms the basis for a multiplicity of different cell signaling phenomena.

Recent studies have suggested that all members of the GPCR superfamily have a conserved structure. Comparisons of avian and mammalian beta-adrenergic receptor cDNA's (Yarden et al., *Proc. Natl. Acad. Sci. USA* 83: 6795-6799, 1986; Dixon et al., *Nature* 321:75-79, 1986; and Kobilka et al., *Proc. Natl. Acad. Sci. USA* 84:46-50, 1987), a bovine rhodopsin cDNA (Nathans and Hogness, *Cell* 34:807-814, 1983), an alpha 2-adrenergic receptor (Kobilka et al., *Science* 238:650-656, 1987), an angiotensin receptor cDNA (Young et al., *Cell* 45:711-719, 1986; Jackson et al., *Nature* 335:437-439, 1988), a bovine substance K receptor (Masu et al., *Nature* 329:836-838, 1987), and a muscarinic acetylcholine receptor cDNA (Kubo et al., *Nature* 323:411-416, 1986) predict that all GPCR share a highly conserved presence of seven hydrophobic transmembrane domains that are suggested to be transmembrane helices of 20-30 amino acids connected by extracellular or cytoplasmic loops. Kobilka et al., *Science* 240: 1310 (1988); Maggio et al., *FEBS Lett.* 319: 195 (1993); Maggio et al., *Proc. Natl. Acad. Sci. USA* 90: 3103 (1993); Ridge et al., *Proc. Natl. Sci. USA* 91, 3204 (1995); Schonenberg et al., *J. Biol. Chem.* 270: 18000 (1995); Huang et al., *J. Biol. Chem.* 256: 3802 (1981); Popot et al., *J. Mol. Biol.* 198: 655 (1987); Kahn and Engelman, *Biochemistry* 31: 6144 (1992); Schoneberg et al. *EMBO J.* 15: 1283 (1996); Wong et al., *J. Biol. Chem.* 265: 6219 (1990); Monnot et al., *J. Biol. Chem.* 271: 1507 (1996); Gudermann et al., *Annu. Rev. Neurosci.* 20: 399 (1997); Osuga et al., *J. Biol. Chem.* 272: 25006 (1997); Lefkowitz et al., *J. Biol. Chem.* 263:4993-4996, 1988; Panayotou and Waterfield, *Curr. Opinion Cell Biol.* 1:167-176, 1989. These transmembrane domains of G-protein coupled receptors are designated TM1, TM2, TM3, TM4, TM5, TM6 and TM7. TM4, TM5, TM6 and TM7 are the most highly conserved and are postulated to provide sequences which impart biological activity to GPCRs. TM3 is also implicated in signal transduction.

The coupling of GPCRs to intracellular signaling molecules such as adenylyl cyclase (Anand-Srivastava et al., *J. Biol. Chem.* 271: 19324-19329 (1996)) and G-proteins (Merkouris et al., *Mol. Pharmacol.* 50: 985-993 (1996)) is reportedly



inhibited by peptides corresponding to the intracellular loops of the receptors. Those studies were conducted primarily to provide an understanding of molecular mechanisms of receptor function and could not be applied directly for drug design, because of the difficulties in intracellular delivery of the inhibitors.

5 WO 94/05695 and U.S. Patent No. 5,508,384 set forth sequences of transmembrane regions for 74 GPCRs. The WO 94/05695 patent publication describes and claims polypeptides corresponding to fragments or homologous sequences of GPCRs which can bind a GPCR ligand or which can modulate ligand binding. Both references disclose that a membrane spanning fragment of the third  
10 TM domain of the dopamine D<sub>2</sub> receptor specifically bound a ligand of the intact receptor in a simple, small unilamellar vesicle model. The fragment used was terminated with a lysine (which is positively charged at physiological pH) at one end and with an aspartic acid (which is negatively charged at physiological pH) at the other. This peptide would not be expected to insert readily into a biological  
15 membrane.

### SUMMARY OF THE INVENTION

The invention generally comprises peptide or peptidomimetic compounds that modulate, and preferably inhibit the biological properties and  
20 activities of GPCRs, by targeting the transmembrane portions of these receptors. The present invention specifically comprises methods for disrupting GPCR function by using these GPCR antagonists.

The present invention provides for the use of chemical or recombinant DNA technology to obtain GPCR polypeptides, which preferably are as small as  
25 possible while still retaining sufficiently high affinity for binding to, or association with, GPCRs. Non-limiting examples of GPCR polypeptides include fragments of 10 to 50 amino acids corresponding to at least one transmembrane domain of domains 1-7. The following are nonlimiting examples of GPCR peptides with antagonist properties.

30 From the GPCR CXCR4

F-2-2: LLFVITLPFWAVDAVANWYFGNDD

F-2-5: LLFVITLPFWAVDAVANDD-OH

F-4-2: VYVGWIPALLLTIPDFIFANDD-OH

F-6-1: VILILAFFACWLPYYIGISID-OH

F-7-3: DDEALAFFHCCLNPILYAFL-NH<sub>2</sub>

F-7-4: DDSITEALAFFHCCLNPILYAFL-NH<sub>2</sub>

5

From the GPCR CCR5

CCR5-TM-2-2: LFFL LTVPFWAHYAAAQWDFGDD

CCR5-TM-4-1: FGVVTSVITWVVAVFASLPGIIFTSSDD

CCR5-TM-6-1: LIFTIMIVYFLFWAPYNIVLLLNTFQED

10 CCR5-TM-7-1: DDQAMQVTETLGMTHCCINPIIYAFV

From the GPCR CCR2

CCR2-TM-2-1: IYLLNLAISDLLFLITLPLWADD-OH

CCR2-TM-2-2: LLFLITLPLWAH SAANEWVFGNDD-OH

15 CCR2-TM-4-1: FGVVTSVITWLVAVF ASVPGIIFTDD

CCR2-TM-6-1: VIFTIMIVYFLFWTPYN IVILLNTFQED

CCR2-TM-7-1: DDATQVT ETLGMTHCCINPIIYAFV

From the GPCR CCR3

20 CCR3-TM-2-1: LLFLVTLPFW IHYVRGHNWVFGDDD

CCR3-TM-4-1: FGVITSIVTWGLAVLAALPEFI FYETED

CCR3-TM-6-1: IFVIMAVFFI FWTPYNVAILLSSYQSDD

CCR3-TM-7-1: DDLVMLVTEVIAYSHCCMNPVIYAFV

25 From the GPCR CCKAR

CCKAR-TM-1-6: DDEWQSALQILLYSIIFLLSVLGNTLVITV

CCKAR-TN-2-1: FLLSLAVSDLMLCLFCMPFNLP

CCKAR-TM-2-2: FLLSLAVSDLMLCLFCM PFNLIDD

CCKAR-TM-6-4: IVVLFFLCWMPIFSANAWRAYDTVDD

30

One embodiment of the invention is an isolated G protein-coupled receptor (GPCR)-modulating molecule comprising a peptide or peptidomimetic that is a structural analog of a portion of a transmembrane domain of a GPCR, wherein said

molecule has a first end and a second end and said molecule has at said first end a negatively charged group and at said second end a neutral charge under physiological conditions; said molecule spontaneously inserts into a membrane in the same orientation as the transmembrane domain from which it is derived; and said molecule modulates a biological property or activity of said GPCR.

In a particular embodiment, the molecules contain a hydrophilic, negatively charged non-peptidic head group and an uncharged tail, which assures correct orientation of the molecule in the cell membrane. In another embodiment, the negatively charged head group is one or more acidic amino acids.

Another embodiment is an isolated GPCR-modulating molecule comprising a peptide or peptidomimetic that is a structural analog of a portion of a transmembrane domain of CXCR4, wherein said portion of said transmembrane domain has a sequence selected from the group of sequences consisting of:

LLFVITLPFWAVDAVANWYFGNDD,  
LLFVITLPFWAVDAVANDD-OH,  
VYGVWIPALLLTIPDFIFANDD-OH,  
VILILAFFACWLPHYIGISID-OH,  
DDEALAFFHCCLNPILYAFL-NH<sub>2</sub>,  
DDSITEALAFFHCCLNPILYAFL-NH<sub>2</sub>,

wherein said molecule modulates a biological activity of said CXCR4. The CXCR4 activity modulated by said peptide includes inhibition of CXCR4-mediated intracellular Ca<sup>2+</sup> release and inhibition of CXCR4-mediated HIV infection.

The invention also comprises methods of modulating the biological activity of a target GPCR by contacting a cell that expresses said GPCR with a molecule of the invention. In one method, the target GPCR is CXCR4, CCR5 or CCR2, and the modulated biological activity is inhibition of GPCR-mediated HIV infection. In another method, the target GPCR is CXCR4 and the modulated biological activity is inhibition of CXCR4-mediated intracellular Ca<sup>2+</sup> release.

Another embodiment is a method of inhibiting HIV-1 infection, comprising contacting a cell that expresses a GPCR that binds HIV-1 with a molecule that comprises a peptide or peptidomimetic that is a structural analog of a portion of

a transmembrane domain of said GPCR, wherein contacting the cell with said molecule inhibits HIV-1 infection. The peptide or peptidomimetic may be a structural analog of a portion of a transmembrane domain of CXCR4 or CCR5.

Peptides corresponding to TM regions of CXCR4, a GPCR that functions as a co-receptor during the cell entry of HIV, were designed and tested in cells, and yielded potent inhibition of HIV entry without apparent toxicity to the cells.

The usefulness of the method is demonstrated by specifically targeting the CXCR4 that functions as a co-receptor during the cell entry of T-cell tropic strains of HIV-1. Peptides containing 20-25 amino acid residues inhibited receptor signaling and HIV-1 infection *in vitro* at concentration as low as 0.2 micromolar.

In one embodiment, the molecules of the present invention mimic a transmembrane domain of the chosen receptor and block self-assembly of that receptor, possibly by competitive inhibition with the native TM domain. They thereby block or inhibit signal transduction in the affected cell.

The invention also includes peptide analogs and peptidomimetics which possess beneficial properties such as increased half-life, lack of immunogenicity, and the ability to cross the blood-brain barrier.

The peptide analogs of the invention mediate the chemical and/or biological effects of hormone agonists/antagonists or other peptides. They are believed to be useful for the development of pharmaceutical, therapeutic, and diagnostic techniques. Accordingly, the invention also provides methods for producing a prophylactic or therapeutic response in a mammal by administering to the mammal a pharmaceutically effective amount of one or more peptide analogs of the invention. In preferred embodiments, the present invention provides methods for producing such responses by modulating the activity of at least one mammalian G-protein-linked receptor by administering an effective amount of one or more peptide analogs of the invention.

In another embodiment, a peptide of the invention may modulate the biological activity of more than one GPCR. In another embodiment, more than one peptide of the invention are administered as a cocktail to modulate the biological activity of more than one GPCR.

### BRIEF DESCRIPTION OF FIGURES

**Figur 1.** Anti-HIV efficacy and toxicity of F-2-2 assay. CEM-SS cells were infected with the LAV strain of HIV-1.

5 **Figure 2.** Anti-HIV efficacy and toxicity of F-4-2 in cytoprotection. CEM-SS cells were infected with the RF strain of HIV-1, which causes cell death, if the inhibitor of infection is not present.

**Figure 3.** The proposed model of transmembrane antagonists action.

10

### DEFINITIONS

A "G-protein" is any member of the superfamily of signal transducing guanine nucleotide binding proteins.

15 A "G-protein-coupled receptor" is any member of a superfamily of receptors that mediates signal transduction by coupling with a G protein. Examples of such receptors include, but are not limited to: CC chemokine receptor 5 (CCR5), CXC chemokine receptor (CXCR4) cholecystokinin type A receptor (CCKAR), adenosine receptors, somatostatin receptors, dopamine receptors, muscarinic cholinergic receptors, alpha-adrenergic receptors, beta-adrenergic receptors, opiate  
20 receptors, cannabinoid receptors, growth hormone releasing factor, glucagon, cAMP receptors, serotonin receptors (5-HT), histamine H2 receptors, thrombin receptors, kinin receptors, follicle stimulating hormone receptors, opsins and rhodopsins, odorant receptors, cytomegalovirus GPCRs, histamine H2 receptors, octopamine receptors, N-formyl receptors, anaphylatoxin receptors, thromboxane receptors, IL-8  
25 receptors, platelet activating factor receptors, endothelin receptors, bombesin gastrin releasing peptide receptor, neuromedin B preferring bombesin receptors, vasoactive intestinal peptide receptors, neurotensin receptors, bradykinin receptors, thyrotropin-releasing hormone receptors, substance P receptors, neuromedin K receptors, renal angiotensin II type I receptors, mas oncogene (angiotensin) receptors  
30 lutropin-choriogonadotropin receptors, thyrotropin receptors, follicle stimulating hormone receptors, cannabinoid receptors, glucocorticoid-induced receptors, endothelial cell GPCRs, testis GPCRs, and thoracic aorta GPCRs, and homologs thereof having a homology of at least 80% with at least one of transmembrane

domains 1-7, as described herein. See, e.g., Probst et al, *DNA and Cell Biology* 11: 1-20 (1992), which is entirely incorporated herein by reference. The term further encompasses subtypes of the named receptors, and mutants and homologs thereof, along with the DNA sequences encoding the same.

5           The term "membrane" refers generally to a lipid bilayer. Preferably, the lipid bilayer is the plasma membrane that delimits a cell, but may be any cellular membrane. The term membrane also encompasses bilayer structures, such as artificial liposomes.

10           The term "GPCR polypeptide" includes polypeptides having an amino acid sequence which substantially corresponds to at least one 10 to 50 (e.g., 10, 20, 25 30 residues) amino acid fragment and/or homologous sequence of a known GPCR or group of GPCRs, wherein the GPCR polypeptide has homology of at least 80%, such as 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92 93, 94, 95, 96, 97, 98, 99 or 100% homology, while maintaining GPCR modulating activity, wherein a GPCR  
15           polypeptide of the present invention is not naturally occurring or is naturally occurring but is in a purified or isolated form which does not occur in nature.

          Preferably, a GPCR polypeptide of the present invention substantially corresponds to a transmembrane domain of a GPCRs. Also preferred are GPCR polypeptides wherein the GPCR amino acid sequence is 4-10 to 50 amino acids in  
20           length, such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acids, or any range therein.

          The term "spontaneously inserts into a membrane" means that a peptide that is brought into contact with a membrane will, under physiological  
25           conditions, arrange itself within the lipid bilayer such that the hydrophobic portion of the peptide is within the membrane, and any charged end is exposed to either surface of a membrane. Preferably, molecules of the present invention that have a net negative charge at one end will orient themselves so that the charged end faces the extracellular surface of the cell.

30           The term "tumor cell" or "cancer cell" or "neoplastic cell" denotes a cell that demonstrates inappropriate, unregulated proliferation. A cell line is said to be "malignant" if, when the cell line is injected into a host animal, the host animal develops tumors or cancers that are anaplastic, invasive, and/or metastatic. A

"human" tumor is comprised of cells that have human chromosomes. Such tumors include those in a human patient, and tumors resulting from the introduction of a human malignant cell line into a non-human host animal if cells from such tumors have human chromosomes.

5           The terms "treating cancer", "cancer therapy", and the like mean generally a treatment that causes any improvement in a mammal having a cancer wherein the improvement is due to treatment with a peptide of the invention. The improvement can be either subjective or objective. For example, if the mammal is human, the patient may note improved vigor or vitality or decreased pain as  
10       subjective symptoms of improvement or response to therapy. Alternatively, the clinician may notice a decrease in tumor size or tumor burden based on physical exam, laboratory parameters, tumor markers, or radiographic findings.

          The phrase "inhibiting tumor [or cell] growth" generally means that the rate of increase in mass, size, number and/or the metabolism of treated cells and/or  
15       tumors is slower as a result of treatment than that of nontreated cells and/or tumors. The growth of a cell line or tumor is said to be "inhibited" by a treatment if, when assayed by means such as radioisotope incorporation into the cells, the treated cells increase in number at a rate that is less than the proliferation rate of untreated control cells, and preferably less than about 50% of the untreated cell proliferation  
20       rate. More preferably, the growth rate is inhibited by at least 80%. If growth is assayed by a means such as plating in methylcellulose, the growth of a cell line is said to be "inhibited" if the treated cells give rise to less than the number of colonies that grow from a like number of untreated cells. Preferably, the number of colonies from treated cells is less than about 70% of the number from untreated cells. More  
25       preferably, the number of colonies is decreased by at least 50%. "Inhibition of cell growth" also encompasses zero growth and, most importantly, consequent death of the tumor cells and eradication of the tumor. When measured *in vivo*, "inhibition of tumor growth" encompasses fewer or smaller tumors (for example, smaller diameter) as compared to control animals or untreated patients. Progression of a tumor refers  
30       to events other than growth, such as morphological and physiological changes, and changes in gene and protein expression.

          Inhibition can be evaluated by any accepted method of measuring whether growth or size of the tumor and/or increase in the number of cancerous or

tumor cells has been slowed, stopped, or reversed. This includes direct observation and indirect evaluation such as subjective symptoms or objective signs. The clinician may notice a decrease in tumor size or tumor burden (number of tumors) based on physical exam, laboratory parameters, tumor markers, or radiographic findings.

Alternatively, if the mammal is human, the patient may note improved vigor or vitality or decreased pain as subjective symptoms of improvement or response to therapy. Some laboratory signs that the clinician may observe for response to therapy include normalization of tests such as white blood cell count, red blood cell count, platelet count, erythrocyte sedimentation rate, and various enzyme levels such as transaminases and hydrogenases. Additionally, the clinician may observe a decrease in a detectable tumor marker such as prostatic specific antigen (PSA) or chorio embryonic antigen (CEA). Alternatively, other tests can be used to evaluate objective improvement such as sonograms, computerized axial tomography scans, nuclear magnetic resonance scans and positron emission testing.

The term "GPCR transmembrane peptide" can include a GPCR transmembrane domain fragment and/or a homologous peptide thereof, of at least 4-50, and preferably 4-30, and preferably at least 10-30 amino acids in length, such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acids, or any range therein, or any corresponding sequences having conservative amino acid substitutions. Sample transmembrane peptides of the invention include, but are not limited to, the peptides listed in Table I of the present disclosure. A preferred transmembrane peptide of the present invention, when contacted with a cell or membrane structure (e.g., liposome) that contains a biologically active GPCR, modulates the biological activity of said GPCR *in vitro*, *in vivo* or *in situ*. The concentration of the peptide in a solution that contacts the cell *in vivo* (e.g, blood plasma or interstitial fluid) or *in vitro* (e.g., culture medium) is between 1 nanomolar and 50 micromolar, preferably between 1 nanomolar and 1 micromolar, and most preferably less than 5 micromolar.

The term "residue" refers to an amino acid or amino acid mimetic incorporated in a oligopeptide by an amide bond or amide bond mimetic.

"Negatively charged" refers to those amino acids, amino acid derivatives, amino acid mimetics and chemical moieties that are negatively charged



at physiological pH. Negatively charged amino acids include, for example Asp and Glu. An "acidic" residue is a residue that is negatively charged at physiological pH.

"Positively charged" refers to those amino acids, amino acid derivatives, amino acid mimetics and chemical moieties that are positively charged at physiological pH. Positively charged amino acids include, for example, Lys and Arg. A "basic residue" is a residue that is positively charged at physiological pH.

"Neutral" refers to those amino acids, amino acid derivatives, amino acid mimetics and chemical moieties that are neither positively nor negatively charged at physiological pH.

"Consensus" sequence refers to peptides which are distinct from known GPCR sequences in critical structural features, but which are derived from consensus sequences of homologous GPCR transmembrane domains 1-7. Such consensus peptides may be derived by molecular modeling, optionally combined with hydrophobicity analysis and/or fitting to model helices, as non-limiting examples...

Such modeling can be accomplished according to known method steps using known modeling algorithms, such as, but not limited to, ECEPP, INSIGHT, DISCOVER, CHEM-DRAW, AMBER, FRODO and CHEM-X. Such algorithms compare transmembrane domains between related G-protein coupled receptors, determine probable energy-minimized structures and define alternative consensus polypeptide fragments.

An amino acid or nucleic acid sequence of a GPCR polypeptide of the present invention is said to "substantially correspond" to another amino acid or nucleic acid sequence, respectively, if the sequence of amino acids or nucleic acid in both molecules provides polypeptides having biological activity that is substantially similar, qualitatively or quantitatively, to the corresponding fragment of at least one GPCR transmembrane domain, or which may be synergistic when two or more transmembrane domains, consensus sequences or homologs thereof are present.

Additionally or alternatively, such "substantially corresponding" sequences of GPCR polypeptides include conservative amino acid or nucleotide substitutions, or degenerate nucleotide codon substitutions wherein individual amino acid or nucleotide substitutions are well known in the art.

The term "modulates a biological property or activity" means that in the presence of a test transmembrane peptide a measurable biological parameter or event

is increased or decreased relative to a control in the absence of said peptide. Examples of biological property or activity include: the conformation of the GPCR, association of the GPCR with other molecules, signal transduction, extracellular secretion of cellular proteins, conformational changes in proteins, changes in enzymatic activity, changes in metabolic activity, changes in affinity for a ligand, changes in levels of viral infection, changes in vasodilation, modulation of heart rate, modulation of bronchodilation, modulation of endocrine secretions and modulation of gut peristalsis. Note that the GPCR biological activity need not be one that is limited to the precise *in vivo* role performed by the GPCR. The term also covers GPCR properties, such as viral protein binding, that are not part of the *in vivo* biological role of the GPCR. It further covers intrinsic properties of GPCRs that are only disclosed by experimental manipulation in the laboratory, such as the ability of GPCRs in artificial bilayers (e.g., liposomes) to interact with GPCR ligands.

"Signal transduction" is the process by which binding of a ligand to a receptor is translated into physiological change. In general, binding of a ligand to a receptor causes a change in a physical property of the receptor, for example a change in its conformation, or its orientation, or in its ability to bind other ligands. This change in a physical property can result, directly or indirectly, in increased or decreased ion fluxes, increased or decreased enzymatic activity, increased or decreased phosphorylation, increased or decreased translocation of the receptor or of any molecule (e.g., an inositol moiety or a G protein subunit) from one cellular compartment to another.

"GPCR ligands" refers to biological molecules that bind GPCRs *in vitro*, *in situ* or *in vivo*, and may include hormones, neurotransmitters, viruses or receptor binding domains thereof, G proteins, opsins, rhodopsins, nucleosides, nucleotides, coagulation cascade factors, odorants or pheromones, toxins, colony stimulating factors, platelet activating factors, neuroactive peptides, neurohumor, or any biologically active compounds, such as drugs or synthetic or naturally occurring compounds.

The phrase "inhibits HIV infection" means that a peptide of the invention inhibits binding of an HIV to a GPCR or inhibits a GPCR biological activity that mediates the entry and successful reproduction of an HIV virus into a GPCR-expressing cell.

The term "effective amount" means a dosage sufficient to produce a desired result. The desired result can be subjective or objective changes in the biological activity of a GPCR, especially signal transduction. Effective amounts of the GPCR polypeptide or composition, which may also include a functional derivative thereof, are from about 0.01 micrograms to about 100 mg/kg body weight, and preferably from about 10 micrograms to about 50 mg/kg body weight, such as 0.05, 0.07, 0.09, 0.1, 0.5, 0.7, 0.9, 1, 2, 5, 10, 20, 25, 30, 40, 45, or 50 mg/kg.

A "conservative substitution", when describing a protein refers to a change in the amino acid composition of the protein that does not substantially alter the protein's activity. Thus, "conservatively modified variations" of a particular amino acid sequence refers to amino acid substitutions of those amino acids that are not critical for protein activity or substitution of amino acids with other amino acids having similar properties (e.g., acidic, basic, positively or negatively charged, polar or non-polar, etc.) such that the substitutions of even critical amino acids do not substantially alter activity. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such substitutions preferably are made in accordance with the following list, which substitutions may be determined by routine experimentation provide modified structural and functional properties of a synthesized polypeptide molecule, while maintaining the receptor binding, or inhibiting or mimicking biological activity, as determined by known GPCR receptor activity assays.

Original Residue	Exemplary Substitution
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Ala	Gly;Ser
Arg	Lys
Asn	Gln;His

Original Residue	Exemplary Substitution
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Asp	Glu
Cys	Se
Gln	Asn
Glu	Asp

	Gay	Ala;Pro
	His	Asn;Gln
	Ile	Leu;Val
	Leu	Ile;Val
5	Lys	Arg;Gln;Glu
	Met	Leu;Tyr;Ile
	Phe	Met;Leu;Tyr
	Se	Thr
	Original	Exemplary
10	Residue	Substitution
	Thr	Se
	Trp	Tyr
	Tyr	Trp;Phe
	Val	Ile;Leu
15		

Put differently, the following six groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 20 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

25 See also, Creighton (1984) PROTEINS, W.H. Freeman and Company. In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variations".

30 The term "substantial identity" or "substantial similarity" in the context of a polypeptide indicates that a polypeptides comprises a sequence which can have 40% sequence identity to a reference sequence, or preferably 70%, or more preferably 85% sequence identity to the reference sequence, or most preferably 90% identity over a comparison window of about 10-20 amino acid residues. "Percentage

amino acid identity" or "percentage amino acid sequence identity" refers to a comparison of the amino acids of two polypeptides which, when optimally aligned, have approximately the designated percentage of the same amino acids. For example, "95% amino acid identity" refers to a comparison of the amino acids of two polypeptides which when optimally aligned have 95% amino acid identity.

Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Because the substituted amino acids have similar properties, the substitutions do not change the functional properties of the polypeptides. An indication that two polypeptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a polypeptide is substantially identical to a second polypeptide, for example, where the two peptides differ only by a conservative substitution. An indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (see generally Ausubel et al., *supra*).

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35: 351-360 (1987). The method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., *J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to

zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

The term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g. degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzner et al., *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka et al., *J. Biol. Chem.* 260:2605-2608 (1985); and Cassol et al., 1992; Rossolini et al., *Mol. Cell. Probes* 8: 91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

The phrase "a nucleic acid sequence encoding" refers to a nucleic acid which contains sequence information for a structural RNA such as rRNA, a tRNA, or the primary amino acid sequence of a specific protein or peptide, or a binding site for a trans-acting regulatory agent. This phrase specifically encompasses degenerate codons (*i.e.*, different codons which encode a single amino acid) of the native sequence or sequences which may be introduced to conform with codon preference in a specific host cell.

"Nucleic acid probes" may be DNA or RNA fragments. DNA fragments can be prepared, for example, by digesting plasmid DNA, or by use of PCR, or synthesized by either the phosphoramidite method described by Beaucage and Carruthers, *Tetrahedron Lett.* 22:1859-1862 (1981), or by the triester method according to Matteucci, *et al.*, *J. Am. Chem. Soc.*, 103:3185 (1981), both incorporated herein by reference. A double stranded fragment may then be obtained, if desired, by annealing the chemically synthesized single strands together under appropriate conditions or by synthesizing the complementary strand using DNA polymerase with an appropriate primer sequence. Where a specific sequence for a nucleic acid probe is given, it is understood that the complementary strand is also identified and included. The complementary strand will work equally well in situations where the target is a double-stranded nucleic acid.

The terms "substantial identity" or "substantial sequence identity" as applied to nucleic acid sequences and as used herein and denote a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, and more preferably at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence.

The phrase "specifically binds to an antibody" or "specifically immunoreactive with", when referring to a protein or peptide, refers to a binding reaction which is determinative of the presence of the protein in the presence of a



heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) *ANTIBODIES, A LABORATORY MANUAL*, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

### DETAILED DESCRIPTION

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the contents of the references cited within the references cited herein are also entirely incorporated by reference.

The present invention is based partly on evidence that transmembrane domains (TM) of GPCRs interact in a specific way in the assembly of receptor molecules. These interactions do not lead to a rigid structure, because some flexibility is required to allow for conformational changes to be made following ligand binding in order to provide the ability of the molecule to signal from the cell surface to the intracellular parts. It was also demonstrated for several GPCRs that the transmembrane domains are involved in ligand binding and thus contain openings that allow penetration of the ligands. Reports that expression of missing transmembrane domains rescues inactive truncated V2 vasopressin, beta-adrenergic and muscarinic M3 receptors (Schoneberg et al. *EMBO J.* 15: 1283 (1996); Wong et al., *J. Biol. Chem.* 265: 6219 (1990); Monnot et al., *J. Biol. Chem.* 271: 1507 (1996); Gudermann et al., *Annu. Rev. Neurosci.* 20: 399 (1997); Osuga et al., *J. Biol. Chem.* 272: 25006 (1997)) suggested peptide derived from the sixth transmembrane domain of P2-adrenergic receptor was found to inhibit receptor activation and dimerization

(Hebert et al., *J. Biol. Chem.*, 271(27):16384-92 (1996)). All these observations suggested to us that targeting intramembrane interactions of GPCRs can specifically regulate GPCR function.

5 The hydrophobic nature of the transmembrane peptides makes their penetrations into the bilayer highly probable. Orientation inside the membrane can be controlled by addition of charged residues to the terminus that is supposed to be extracellular.

#### 1. GPCR peptides

10 GPCR polypeptides of the present invention, or nucleic acids encoding therefor, include a finite set of substantially corresponding sequences as substitution peptides or polynucleotide which can be routinely obtained by one of ordinary skill in the art, without undue experimentation, based on the teachings and guidance presented herein. For a detailed description of protein chemistry and structure, see Schulz et al., *PRINCIPLES OF PROTEIN STRUCTURE*, Springer-Verlag, New York, 1978, 15 and Creighton, T. E., *PROTEINS: STRUCTURE AND MOLECULAR PROPERTIES*, W. H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. For a presentation of nucleotide sequence substitutions, such as codon preferences, see Ausubel et al, *supra*, at sections A.1.1-A.1.24, and Sambrook et al., *supra*, at Appendices C and D.

20 GPCR polypeptides include homologous sequences and/or fragments of at least one of transmembrane domain 1-7 of one or more GPCRs or homologs thereof, which GPCR polypeptides do not occur naturally, and/or which are provided in an isolated and/or purified form not found in nature.

25 However, in the context of the present invention, GPCR polypeptides of greater than 15-20 amino acids are preferred such that the GPCR polypeptides are able to span the lipid bilayer.

30 It is particularly preferred that peptides of the invention be selected or modified so that one end is charged and the other is neutral under physiological conditions. This is so that the peptide spontaneously inserts into a membrane. It is of particular importance that the peptide insert in the same orientation as the transmembrane GPCR domain from which it is derived.

Peptides of the invention can be derived from any of the 7 TM domains. Non-limiting, illustrative examples of GPCR TM1 and TM2 transmembrane

domains that are used to generate molecules of the present invention include the following:

		101	TM1	150
5	GPCRAelegans	GPCEDIMGYV	WLTVVSEFMVG	ALVLLTSQ... .RRLN
	GRH	NLPTLTLSGK	IRVTVTFFLF	LLSATFNASF LLKLQKWTK KEKGKKLSRM
	TRH	RAVVALEYQV	VTILLVLIIC	GLGIVGNIMV VLVVMR... .TKHMRTP
	FSHprec	NPCEDIMGYN	ILRVLIWFIS	ILAITGNIIV LVILTTTSQYK .....LTV
	TSHprec	NPCEDIMGYK	FLRIVVWFVS	LLALLGNVFN LLILLTSHYK .....LNV
10	LH_CGprec	NPCEDIMGYD	FLRVLIWLIN	ILAIMGNMTV LPVLLTSRY. ....KLT
	PGE_EP1	PNTSAVPPSG	ASPALPIFSM	TLGAVSNLLA LALLAQAA.G RLRRRSATT
	PGE_EP2	SASLSPDRLN	SPVTIPAVMF	IFGVVGNLVA IVVLCKS... .RKEQKETT
	PGE_EP3	QWLPPGE...	SPAISSVMF	SAGVLGNLIA LALLARRW.R SAGRSSLSL
	PGF	SNTTCQTENR	LSVFFSVIFM	TVGILSNLSA IAILMKAY.Q RFRQKSKA.S
15	PGI	CRNLTYVRGS	VGPATSTLMF	VAGVVGNGLA LGILSARRPA RP.....SA
	TXA2	NITLEERRLI	ASPWFAASFC	VVGLASNLLA LSVLAGA... ROGGSHTRSS
	PAF	HMDSEFRYTL	F.PIVYSIIF	VLGVIANGYV LWVFARLY.P CKKFNEIK..
	M2	...YKTFEVV	FIVLVAGSL	LVTIIGNILV MVS.I.KVN.R HLQT.....V
	M4	HNRYETVEMV	FIATVTGSL	LVTVVGNILV MLSI.KVN.R QLQT.....V
20	M1	..GKGPWQVA	FIGITTGLS	LATVTGNLLV LISF.KVN.T ELKT.....V
	M3	LGGHTVWQVV	FIAFLTGILA	LVTIIGNILV IVSF.KVN.K QLKT.....V
	M5	LERHRLWEVI	TIAAVTAVVS	LITIVGNVLV MISF.KVN.S QLKT.....V
	H1	KTTMASPOLM	PLVVVLSTIC	LVTVGLNLLV LYAVRSE... KLHT.....V
	H2	FCLDSTACKI	TITVVLAVLI	LITVAGNVVV CLAVGLNR.. RLNR.....L
25	5HT1A	ISDVTVSQV	ITSLLLGLTI	FCAVLGNACV VAAIALER.. SLQN.....V
	5HT1B	QDSISLPWKV	LLVMLLALIT	LATTLNNAFV IATVYRTR.. KLHT.....P
	5HT1D	DPRTLQALKI	SLAVVLSVIT	LATVLSNAFV LTTILLTR.. KLHT.....P
	5HT1E	IRPKTITEKM	LICMTLVVIT	TLTLLNLAV IMAIGTTK.. KLHQ.....P
	5HT1F	ELLNRMPSKI	LVSLLTSLGLA	LMTTINSLV IAAIIVTR.. KLHH.....P
30	5HT2A	QEK.....	WSALLTAVVI	ILTIAGNILV IMAVSLEK.. KLQN.....A
	5HT2B	IVEEQGNKLH	WAALLILMVI	IPTIGGNTLV ILAVSLEK.. KLQV.....A
	5HT2C	..QN.....	WPALSIVIII	IMTIGGNILV IMAVSMEX.. KLHN.....A
	5HT5A	SSPLLSVFGV	LILTLGLFLV	AATFAWNLV LATILVRV.. TFHR.....V
	5HT5Brat	REPPFSAFTV	LVTLLVLLI	AATFLWNLLV LVTILVRV.. AFHR.....V
35	5HT6rat	GPPFAPGGSG	WVAAALCVVI	VLTAANSLL IVLICTQP.. AVR.....T
	5HT7	QINYGRVEKV	VIGSILTLIT	LLTIAGNCLV VISVCFVK.. KLRQ.....P
	alpha1A	GGLVVSAGQV	GVGVFLAAFI	LMAVAGNLLV ILSVACNR.. HLQT.....V
	alpha1B	..QLDITRAI	SVGLVLGAFI	LFAIVGNILV ILSVACNR.. HLRT.....P
	alpha1C	PAPVNISKAI	LLGVILGGLI	LFGVLCNILV ILSVACHR.. HLHS.....V
40	alpha2A	..YSLQVTL	TLVCLAGLLM	LLTVFGNVLV IIAVFTSR.. ALKA.....P
	alpha2B	QDPYSVQATA	AIAAAITFLI	LFTIFGNALV ILAVLTSR.. SLRA.....P
	alpha2C1	RGQYSAGAVA	GLAAVVGFLI	VFTVVGNVLV VIAVLTSR.. ALRA.....P
	alpha2C2	RGQYSAGAVA	GLAAVVGFLI	VFTVVGNVLV VIAVLTSR.. ALRA.....P
	beta1	EPLSQWTAG	M.GLLMALIV	LLIVAGNVLV IVAIAKTP.. RLQT.....L
45	beta2	QORDEVWVVG	M.GIVMSLIV	LAIVFGNVLV ITAIAKFE.. RLQT.....V
	beta3	GLPGVPWEAA	LACALLALAV	LATVGGNLLV IVAIAWTP.. RLQT.....M
	beta4turkey	SWAAVLSRQW	AVGAALSITI	LIVIVAGNLLV IVAIAKTP.. RLQT.....M
	D1A	VVERDFSVRI	LTACFLSLLI	LSTLLGNLTV CAAVIRFR.. HLRSK....V
	D2	DGKADRPHYN	YYATLLTLLI	AVIVFGNVLV CMAVSREK.. ALQT.....T
50	D3	TGASQARPHA	YYALSICALI	LAIVFGNGLV CMAVLKER.. ALQT.....T
	D4	ASAGLAGQGA	AALVGGVLLI	GAVLAGNSLV CVSVATER.. ALQT.....P
	D5	GAPPLGPSQV	VTACLLTLLI	IWTLLGNVLV CAAIVRSR.. HLRAN....M
	A1	MPPSISAFQA	AYIGIEVLIA	LVSVPGNVLV IWAVKVNQ.. ALRD.....A
	A2a	..MPIMGSS	VYITVELAIA	VLAILGNVLV CWAVWLNS.. NLQN.....V
55	A2b	..MLLETQDA	LYVALELVIA	ALSVAGNVLV CAAVGTAN.. TLQT.....P
	A3	NSTTSLANV	TYITMEIFIG	LCAIVGNVLV ICVVKLNP.. SLQT.....T
	OCdrome	LAVPE.WEAL	LTALVLSVII	VLTIIIGNILV ILSVFTYK.. PLRI.....V
	ACTH	RNNSDCPRVV	LPEEIFFTIS	IVGVLENLIV LLAVFKNK.. NLQA.....P
	MSH	QTGARCLEVS	ISDGLFSLG	LVSLVENALV VATIAKNR.. NLHS.....P
60	MC3	SSSAFCEQVF	IKPEIFSLG	IVSLLLENILV ILAVVRNG.. NLHS.....P
	MC4	SDGGCYEQLF	VSPEVFVTLG	VISLLENILV IVAIAKNR.. NLHS.....P
	MC5	NKSSPCEDMG	IAVEVFLTLG	VISLLENILV IGAIVKNK.. NLHS.....P
	melatonin	DGARPSWLAS	ALACVLIFTI	VVDILGNLLV ILSVYRNKKL .....RNA
	oxytocin	R..RNEALAR	VEVAVLCLIL	LLALSGNACV LLALRTTRQK .....HSR
65	conopressinLs	FHGVEDLLK	IEIAVQATIL	YMTLFGNGIV LLVRLRRQK .....LTR
	V1A	RDVRNEELAK	LEIAVLAVTF	AVAVLGNSSV LLAL.....HRTPRKTSR

	V1B	WLGRDEELAK	VEIGVLATVL	VLATGGNLAV	LLTLGQLGRK	.....RSR
	V2	LDTRDPLLAR	AELALLSIVF	VAVALSNGLV	LAALA.....	RRGRRGHWAP
	CCK_A	PRPSKEWQPA	VQILLYSLIF	LLSVLGNTLV	ITVLI.....	RNKR.....RTV
	CCK_B	GAGTRELELA	IRITLYAVIF	LMSVGGNMLI	IVVLGLS...	...RRL...RTV
5	NPY1	DCHLPLAMIF	TLALAYGAVI	ILGVSGNLAL	IIIL.....	KQKEM...RNV
	NTR	DVNTDIYSKV	LVTAVYLALF	VVGTVGNTVT	AFTLAR....	KKSLSQSQST
	NK1	QFVQPAWQIV	LWAAAYTVIV	VTSVVGNVVV	MWILA....	HKRM...RTV
	NK2	AFSMPSWQLA	LWAPAYLALV	LVAVTGNAIV	IWILA....	HRRM...RTV
	NK3	QFVQPSWRIA	LWSLAYGVVV	AVAVLGNLIV	IWILA....	HKRM...RTV
10	blueops	YHIAPVWAFY	LQAAFMGTVF	LIGFPLNAMV	LVATL.....	RYKKL...RQP
	greenops	YHIAPRWVYH	LTSVWMIFVV	IASVFTNGLV	LAATM.....	KFKKL...RHP
	redops	YHIAPRWVYH	LTSVWMIFVV	TASVFTNGLV	LAATM.....	KFKKL...RHP
	rhodopsin	YYLAEPWQFS	MLAAYMFLLI	VLGFPINFLT	LYVTVQ....	HKKL...RTP
	violetopsGg	YHIAPPWAFY	LQTAFMGIVF	AVGTPLNAVAV	LWVTVRYKRL	.....RQP
15	opsin crab	FPPMNPLWYS	ILGVAMIILG	IICVLGNMNV	IYLMMTTKSL	.....RTP
	ET_Aprec	QTKITSAFKY	INTVISITIF	IVGMVGNATL	LRITYQ....	...NKCM...RNG
	ET_Bprec	PIEIKETFKY	INTVVSCLVF	VLGIIGNSTL	LRITYKKN...	...CM...RNG
	ET_Cfrog	RAKIRHAFKY	VTTILSCVIF	LVGIVGNSTL	LRITYKKN...	...CM...RNG
	galanin	PLFGIGVENF	VTLVVFGFLI	ALGVLGNSLV	ITVLARSK...	...PGKPRST
20	NMB	GTTTELIVRC	VIPSLYLLII	TVGLLGNIML	VKIFTNS...	...AM...RSV
	GRP	DDWSHPGILY	VIPAVYGVII	LIGLIGNITL	IKIFCTVK...	...SM...RNV
	BRS3	DNSPGIEALC	AIYITYAVII	SVGILGNAIL	IKVFFKTK...	...SM...QTV
	deltaOP	GSASSLALAI	AITALYSAVC	AVGLLGNVLV	MFGIVRYT...	...KM...KTA
	kappaOP	PAHISPAIPV	IITAVYSVVF	VVGLVGNLNV	MFVIRYT...	...KM...KTA
25	muOP	GSP.SMITAI	TIMALYSIVC	VVGLFGNVLV	MYVIRYT...	...KM...KTA
	OPRX	GAFLPLGLKV	TIVGLYLAVC	VGGLLGNCLV	MYVILRHT...	...KM...KTA
	CB1	FMVLNPSQQL	AIAVLSLTLG	TFTVLENLLV	LCVILHSR...	SLRCR....P
	CB2	YMILSGPQKT	AVAVLCTLLG	LLSALENVAV	LYLILSSH...	QLRRK....P
30	SSTR1	TLSEGQGSAL	LISFIYSVVC	LVGLCGNSMV	IYVILRYA...	...KM...KTA
	SSTR2	EPYYDLTSNA	VLTFIYFVVC	IIGLCGNTLV	IYVILRYA...	...KM...KTI
	SSTR3	SPAGLAVSGV	LIPLVYLVVC	VVGLLGNLNV	IYVILRHT...	...AS...PSV
	SSTR4	GDARAAG.MV	AIQCIYALVC	LVGLVGNALV	IFVILRYA...	...KM...KTA
	SSTR5	PAPSAGARAV	LVPVLYLLVC	AAGLGGNTLV	IYVILRFA...	...KM...KTV
35	IL8A	MLETETLNKY	VVIIAYALVF	LLSLLGNLNV	MLVILYSR...	...VG...RSV
	IL8B	EPESLEINKY	FVVIYALVF	LLSLLGNLNV	MLVILYSR...	...VG...RSV
	AT1a	KAGRHNIFV	MIPTLYSIIF	VVGIFGNSLV	VIVIFYFM...	...KL...KTV
	AT1brat	KAGRHNIFV	MIPTLYSIIF	VVGIFGNSLV	VIVIFYFM...	...KL...KTV
	AT2	QKPSDKH.LD	AIPILYIIF	VIGFLVNIVV	VTLFCCQK...	...GP...KKV
40	BK1	APEAWDLLHR	VLPTFIISIC	FFGLLGNLNV	LLVFLLR...	...RQLNV
	BK2	QVEWLGLWNT	IQPPFLWVLF	VLATLENIFV	LSVFLCHK...	...SSCTV
	P2Y7	PSLGEVFISL	LAIILLSVAL	AVGLPGNSFV	VWSILKRMQ...	...KRSV
	P2Y6	CVYREDFKRL	LLPPVYSVVL	VVGLPLNVCV	IAQICASRR...	...TLTR
	P2Y5	CSTEDSFKYT	LYGCVFSMVF	VGLLIANCVA	IYIFTFTLK...	...VRNE
	P2Y4	CWFDEDFKFI	LLPVSYAVVF	VLGLGLNAPT	LWLFIFRLR...	...PWDA
45	P2Y3chick	CTFHEEFKQV	LLPLVYSVVF	LLGLPLNAVAV	IGQIWLARK...	...ALTR
	P2Y2	CRFNEDFKYV	LLPVSYGVVC	VLGLCLNAVAV	LYIFLCRLK...	...TWN
	P2Y1	ALTKTGQFY	YLPVYILVF	IIGFLGNSVA	IWMFVFMK...	...PWSG
	THRprec	GYLTSWTL	FVPSVYTGVF	VVSLPLNIMA	IVVFILKM...	...KV...KKP
	C5a	TSNTLRVPDI	LALVIFAVVF	LVGLVGNALV	VWVTAFEA...	...K...RTI
50	GP01mouse	AESEPELVVN	PWDIVLCSSG	TLICCENAVV	VLIIF.HSPS	LR.....AP
	R334rat	VESEPELVVN	PWDIVLCSSG	TLICCENAVV	VLIIF.HSPS	LR.....AP
	GP21mouse	GPATLLPSPR	AWDVLCISG	TLVSCENALV	VAIIV.GTPA	FR.....AP
	GCRCmouse	AESQNPTVKA	LLIVAYSFTI	VFSLFGNVLV	CHVIFK.NQR	.....MHSA
55	TXKR	...QPPWAVA	LWSLAYGAVV	AVAVLGNLNV	IWIVLA.HKR	MR.....TV
	G10Drat	MELNENTKQV	VLFVYLAIF	VVGLVENVLV	IC.VNCRRS	R.....VGM
	RDC1	NMPNKSULLY	TLFSIYIFIF	VIGMIANSVV	VW.VNIQAKT	TGYDT.....
	BLR1	...MASFKAV	FVPVAYSLIF	LLGVIGNVLV	LV.ILHRHQ	TRASTE....
	CL5	REENANFNKI	FLPTIYSIIF	LTGIVGNLNV	IL.VMGYQKK	LRSMTDKYR.
60	LCR1	REENANFNKI	FLPTIYSIIF	LTGIVGNLNV	IL.VMGYQKK	LRSMTDKYR.
	EBI1	KKDVRNFKAW	FLPIMYSIIC	FVGLLGNLNV	VL.TYIYFKR	LKMTIDTY..
	RBS1rat	LGDIVAFGTI	FLSIFYSLVF	TFLVGNLNV	VL.ALNSRK	SKSITDIY..
	EBI2	LYAHHSTARI	VMPLHYSLVF	IIGLVGNLLA	LV.VIVQNRK	KINSTTLY..
	GCRTchick	CSTEDSFKYT	LYGCVFSMVF	VGLLIANCVA	IY.IFTFTLK	VRNETTTY..
	APJ	EYTDWKSSGA	LIPAIYMLVF	LLGTTGNLNV	LWTVFRSSRE	KRSAD....
65	RTArat	EQIATLPPPA	VTNYIFLLC	LCGLVGNLNV	LWFFGFSIK.	RT.....P
	UHRrat	SLQLVHQLKG	LIVMLYSIVV	VVGLVGNLNV	VLVIARVR...	...RLHN
	FMRL1	EPAGHTVLWI	FSLLVHGVTF	VFGVLGNLNV	IWVA.GFR...	...MTRTV
	FMRL2	ESAGYTVLRI	LPLVVLGVTF	VGLVGNLNV	IWVA.GFR...	...MTRTV

	fMLP	VSAGYLFLLDI	ITYLVFAVTF	VLGVLGNGLV	IWVA.GFR..	.....MHTTV
	OLF1catfish	NGFYNIPTK	YYYAFLCIAY	AVTVLGNSTFI	MCTIYLAR..	.....SLHTA
	OLF3catfish	TGLYNIPHAK	YYYFLFCFVY	TVTFGLGNSFI	MGTIYLAR..	.....SLHTA
	OLF8catfish	GFHDLGEWGP	ILSIPYLLMF	LLSSTSNLTL	IYLIISQR..	.....ALHSP
5	OLF32Acatfish	SGFSGIPFSQ	YYFAFLIFY	IISLCGNSIV	LFMILVDR..	.....TLHIP
	OLF32Bcatfish	SGFSGIPFSQ	YYFVFLIFY	IISLCGNSIV	LFMILVDR..	.....TLHIP
	OLF32Ccatfish	SGFSGIPFSQ	YYFVFLIFY	IISLCGNSIV	LFMILVDR..	.....TLHIP
	OLF32Dcatfish	SGFSGIPFSQ	YYFVFLIFY	IISLCGNSIV	LFMILVDR..	.....TLHIP
	OLF47catfish	IAYNSLGNKN	YLILALGIY	LITLLCNFTL	LAILMNS..	.....SLQNP
10	OLF202catfish	FPGLPPNYG	LVSVMFFVY	VCTLIGNCTF	FTLFLEK..	.....SLQKP
	OLFCOR1chicken	LTD.NPGLQM	PLFMVFLAIY	TITLLTNLGL	IALISVDL..	.....HLQTP
	OLFCOR2chicken	LTD.NPRLQM	PLFMVFLVIY	TTTLLTNLGL	IALIGMDL..	.....HLQTP
	OLFCOR3chicken	LTD.NPGLQM	PLFMVFLAIY	TITLLTNLGL	IRLISVDL..	.....HLQTP
	OLFCOR4chicken	LTD.NPGLQM	PLFMVFLAIY	TITLLTNLGL	IRLISVDL..	.....HLQTP
15	OLFCOR5chicken	LTD.NPRLQM	PLFMVFLAIY	TITLLTNLGL	IALISVDF..	.....HLQTP
	OLFCOR6chicken	LTD.NPGLQM	PLFMVFLAIY	TITLLTNLGL	IALIRIDL..	.....QLQTP
	OLFdog	LPI.DPDQD	LFYALFLAMY	VTTILGNLLI	IVLIQDLS..	.....HLHTP
	OLF07E	MSE.SPEQQQ	ILFWMFLSMY	LVTVVGNVLI	ILAISSDS..	.....RLHTP
	OLF07I	LPI.QPEQQN	LCYALFLAMY	LTTLLGNLLI	IVLIRLDS..	.....HLHTP
20	OLF07J	FSS.FHEQQI	TLFGVFLALY	ILTLAGNII	VTIIRIDL..	.....HLHTP
	OLFOR3mouse	VSD.HPHLEI	IFFAVILASY	LLTLVGNLTI	ILLRDLA..	.....RLHTP
	OLFrat	LTK.QPELLL	PLFFFLFLVIY	VLTVVGNLGM	ILLIIVSP..	.....LLHTP
	OLFF3rat	FVE.NKDLOP	LIYGLFLSMY	LVTVIGNISI	IVAIISDP..	.....CLHTP
	OLFF5rat	LSR.QPQQQQ	LLFLLFLIMY	LATVLGNLLI	ILAIGTDS..	.....RLHTP
25	OLFF6rat	FPG.PRSMRI	GLFLLFLVMY	LLTVVGNLAI	ISLVGAHR..	.....CLQTP
	OLFF12rat	FTE.NPQLHF	LIFALFLSMY	LVTVLGNLLI	IMAIITQS..	.....HLHTP
	OLFI3rat	LPI.PEEHQH	LFYALFLVMY	LTILGNLLI	IVLVQDLS..	.....QLHTP
	OLFI7rat	FPA.PAPLRV	LLFFLSLLXY	VLVLTENMLI	IIAIRNHP..	.....TLHQP
	OLFI8rat	LPI.PPEHQQ	LFFALFLIMY	LTTLGNLLI	VVLVQDLS..	.....HLHTP
30	OLFI9rat	LPI.PPEYQH	LFYALFLAMY	LTTLGNLLI	IILILDS..	.....HLHTP
	OLFI14rat	LPI.PSEYHL	LFYALFLAMY	LTILGNLLI	IVLVRLDS..	.....HLHMP
	OLFI15rat	LPI.PSEHQH	VFYALFLSMY	LTTLGNLLI	IILIHDS..	.....HLHTP
	OLFOR17_40	LLE.APGLQP	VVFVLFLEY	LVTVRGNLSI	LAALVSEP..	.....KLHTP
	GUST27rat	...MILNCN	PFSGLFLSMY	LVTVLGNLLI	ILAVSSNSHL	.....HNL
35	RPE	PTGFGELEVL	AVGMVLLVEA	LSGLSLNTLT	IFSFKTPEL	.....RTP
	HHRF1	FTDVLNQSKP	VTLFLYGVVF	LFGSIGNFLV	IFTITWRRRI	.....QCS
	HHRF2	NSTEIYQLFE	YTRLGVWLMC	IVGTFLNVLV	ITTILYRRK	K.....KSP
	HHRF3	MTGPLFAIRT	TEAVLNTFII	FVGGPLNAIV	LITQLLTNRV	LG.....YST
	MCP-1A	..DVKQIGAQ	LLPPLYSLVF	IFGFVGNMLV	VLILINCKKL	.....KCL
40	MCP-1B	..DVKQIGAQ	LLPPLYSLVF	IFGFVGNMLV	VLILINCKKL	.....KCL
	PPR1bovine	..EVRKFAKV	FLPAFFTIAF	IIGLAGNSTV	VAIYAYYKRR	.....RTK

## TM2

45	GPCRAelegans	151	TRFLMCNLAF	ADFI GLYIF	ILTSVSAVTR	GDYHNYVQW	QNGAGCKILG
	GRH		.KLLLKHLTL	ANLLETIVM	PLDGMWNITV	QWYA.....	.GELLCKVLS
	TRH		TNCYLVSLAV	ADLMVLVAAG	LPNITDSIYG	SWVGYV...	.....GCLCIT
50	FSHprec		PRFLMCNLAF	ADLCIGIYLL	LIASVDIHTK	SQYHNYAIDW	QTGAGCDAAG
	TSHprec		PRFLMCNLAF	ADFCMGMYLL	LIASVDLYTH	SEYYNHAIIDW	QTGPGCNTAG
	LH_CGprec		PRFLMCNLAF	ADFCMGLYLL	LIASVDSQTK	GQYYNHAIIDW	QTGSGCSTAG
	PGE_EP1		FLLFVASLLA	TDLAGHVIPG	ALVRLRYTA.	.....GRA	PAGGACHEFLG
	PGE_EP2		FYTLVCGLAV	TDLLGTLLVS	PVTIATYMKG	.....QWPG	GQP.LCEYST
	PGE_EP3		FHVLVTELVE	TDLLGTCLIS	PVVLASYARN	QT..LVALAP	ESR.ACTYFA
55	PGF		FLLLASGLVI	TDFFGHLING	AIASFVYASD	KE..WIRFDQ	.SNVLCISIFG
	PGI		FAVLVTGLAA	TDLLGTSFSL	PAFVAYARN	SS..LLGLAR	GCPALCDAFA
	TXA2		FLTFLCGLVL	TDFLGLLVTG	TIVVSQHAAL	FE..WHAVDP	GGR.LCRFMG
	PAP		..IFMVNLTM	ADMLFLITLP	LIVIVYQ.NQ	GNWIL.....	PK.FLCNVAG
	M2		NNYFLFSLAC	ADLIIGVFSM	NLYTLYTVIG	.....YWPL	.GPVVCIDLWL
60	M4		NNYFLFSLAC	ADLIIGAFSM	NLYTVYIIGK	.....YWPL	.GAVVCIDLWL
	M1		NNYFLLSLAC	ADLIIGTFSM	NLYTTYLLMG	.....HWAL	.GTLACDLWL
	M3		NNYFLLSLAC	ADLIIGVISM	NLFTTYIIMN	.....RWAL	.GNLACDLWL
	M5		NNYFLLSLAC	ADLIIGIFSM	NLYTTYILMG	.....RWAL	.GSLACDLWL
	H1		GNLYIVSLSV	ADLIVGAVVM	PMNILYLLMS	.....KWSL	.GRPLCLFWL
65	H2		TNCFIVSLAI	TDLLGLLLVL	PFSAIYQLSC	.....KWSF	G.KVFCNIYT
	5HT1A		ANYLIGSLAV	TDLMSVSLVL	PMAALYQVLN	.....KWTL	.GOVTCDFI
	5HT1B		ANYLIASLAV	TDLLVSILVM	PISTMYTVTG	.....RWTL	.GOVVCDFWL
	5HT1D		ANYLIGSLAT	TDLLVSILVM	PISIAYTITH	.....TNWF	.GOILCDIWL

	5HT1E	ANYLICSLAV	TDLLVAVLVM	PLSIIYIVMD	.....RWKL	.GYFLCEVWL
	5HT1F	ANYLICSLAV	TDFLVAVLVM	PFSIVYIVRE	.....SWIM	.GOVVCDIWL
	5HT2A	TNYFLMSLAI	ADMLLGFLVM	PVSMLTILYG	.....YRWPL	P.SKLCVWI
5	5HT2B	TNYFLMSLAV	ADLLVGLFVM	PIALLTIMFE	.....AMWPL	P.LVLCPAWL
	5HT2C	TNYFLMSLAI	ADMLVGLLVM	PLSLLAILYD	.....YVWPL	P.RYLCPVWI
	5HT5A	PHNLVASMAY	SDVLVAALVM	PLSLVHELSE	....GRRWQL	.GRRLCQLWI
	5HT5Brat	PHNLVASTAV	SDVLVAALVM	PLSLVSELSE	....GRRWQL	.GRSLCHVWI
	5HT6rat	SNFFLVSLFT	SDLMVGLVVM	PPAMLNLYG	.....RWVL	A.RGLCCLLWT
10	5HT7	SNYLIVSLAL	ADLSVAVAVM	PFVSVTDLIG	G.....KWIF	.GHFFCNVFI
	alpha1A	TNYFIVNLAV	ADLLLSATVL	PFSATMEVLG	.....FWAF	G.RAFCDVWA
	alpha1B	TNYFIVNLAM	ADLLLSFTVL	PFSAALEVLG	.....YVWL	G.RIFCDIWA
	alpha1C	THYYIVNLAV	ADLLLTSTVL	PFSAIFEVLG	.....YWAF	G.RVFCNIWA
	alpha2A	QNLFLVSLAS	ADILVATLVI	PFSLANEVMG	.Y.....WYF	.GKAWCEIYL
	alpha2B	QNLFLVSLAA	ADILVATLII	PFSLANELLG	.Y.....WYF	R.RTWCEVYL
15	alpha2C1	QNLFLVSLAS	ADILVATLVM	PFSLANELMA	.Y.....WYF	.GOVWCGVYL
	alpha2C2	QNLFLVSLAS	ADILVATLVM	PFSLANELMA	.Y.....WYF	.GOVWCGVYL
	beta1	TNLFIMSLAS	ADLVMGLLVV	PFGATIVVWG	.....RWEY	GS.FFCELWT
	beta2	TNYFITSLAC	ADLVMGLAVV	PFGAAHILMK	.....MWTF	GN.FWCEFMT
20	beta3	TNVFVTSLAA	ADLVMGLLVV	PPAATLALTG	.....HWPL	GA.TGCELWT
	beta4turkey	TNVFVTSLAC	ADLVMGLLVV	PPGATILLSG	.....HWPL	GT.VVCELWT
	D1A	TNFFVISLAV	SDLLVAVLVM	PWKAVAEIAG	.....FWPF	GS..FCNIWV
	D2	TNYLIVSLAV	ADLLVATLVM	PWVVYLEVVG	E.....WKF	S.RIHCDIFV
	D3	TNYLVVSLAV	ADLLVATLVM	PWVVYLEVTG	GV.....WNF	S.RICCDVVFV
25	D4	TNSFIVSLAA	ADLLLALLVL	PLFVYSEVQG	GA.....WLL	SPRLC.DALM
	D5	TNVFIVSLAV	SDLFVALLVM	PWKAVAEVAG	.....YWPF	GA..FCDVWV
	A1	TFCFIVSLAV	ADVAVGALVI	PLAILINIGP	QTYFHTCL..	.....MVA
	A2a	TNYFVVSLAA	ADIAVGVLAI	PFAITISTGF	CAACHGCL..	.....FIA
	A2b	TNYFLVSLAA	ADVAVGLFAI	PFAITISLGF	CTDFYGCCL..	.....FLA
30	A3	TFYFIVSLAV	ADIAVGVLVM	PLAIVVSLGI	TIHFYSCL..	.....FMT
	OCdrome	QNFFIVSLAV	ADLTVALLVV	PFNVAYSILG	R.....WEF	GI.HLCKLWL
	ACTH	MYFFICSLAI	SDMLGSLYKI	LENILIIILRN	MGYLKPRGSF	ET.TADDIID
	MSH	MYCFICCLAL	SDLLVSGTIV	LETAVILLE	AGALVARAAV	LQ.QLDNVID
	MC3	MYFFLCSLAV	ADMLVSVSNA	LETIMIAIVH	SDDYTFEDQF	IQ.HMDNIFD
35	MC4	MYFFICSLAV	ADMLVSVSNG	SETIIITLLN	STD.TDAQSF	TV.NIDNVID
	MC5	MYFFVCSLAV	ADMLVSMSSA	WETITIIYLLN	NKHLVIADAF	V.RHIDNVFD
	melatonin	GNIFVVSLAV	ADLVVAIYPY	PLVLSMIFNN	GWNLGYLH..	.....CQVSG
	oxytocin	LFFFMKHLSI	ADLVVAVFQV	LPQLLWDITF	RFYGP.....	..DLLCRLVK
	conopressinLs	MQWFIHLAF	ADIFVGFFNI	LPQLISDVTI	VFHGDD....	..FTCRFIK
40	V1A	MHLFIRHLSL	ADLAVAFFQV	LPQMCWDITY	RFRGPD....	..WLCRAVK
	V1B	MHLFVLHLAL	TDLAVALFQV	LPQLLWDITY	RFQGP.....	..DLLCRAVK
	V2	IHFVIGHLCL	ADLAVALLFQV	LPQLAWKATD	RFRGPD....	..ALCRAVK
	CCK_A	TNIFLLSLAV	SDMLCLFCM	PFNLIPNLLK	DFIFGS....	..AVCKTTT
	CCK_B	TNAFLLSLAV	SDLLLAVACM	PFTLLPNLMG	TFIFGT....	..VICKAVS
45	NPY1	TNIIIVNLSF	SDLLVAIMCL	PFTFVYTLMD	HWVFG.....	..AMCKLNP
	NTR	VHYHLGSLAL	SDLLTLLLAM	PVELYNFIWV	HHPWAF....	..GDAGCRGYY
	NK1	TNYFLVNLA	AEASMAAFNT	VVNFTYAVHN	EWYVGL....	..FYCKFHN
	NK2	TNYFIVNLAL	ADLCMAAFNA	AFNFVYASHN	IWYFGR....	..AFCYFQN
	NK3	TNYFLVNLA	SDASMAAFNT	LVNFIYALHS	EWYFGA....	..NYCRFQN
50	blueops	LNYILVNVSF	GGFLLCIPSV	FPVFVASCNG	YFVFG.....	..HVCALG
	greenops	LNWILVNLA	ADLAETVIAS	TISVVNQVYG	YFVLGH....	..PMCYLEG
	redops	LNWILVNLA	ADLAETVIAS	TISIVNQVSG	YFVLGH....	..PMCYLEG
	rhodopsin	LNYILLNLAV	ADLFMVLGGF	TSTLYTSLHG	YFVFGP....	..TGCNLEG
	violetopsGg	LNYILVNISA	SGFVSCVLSV	FVVFVASARG	YFVFG.....	..KRVCELEA
55	opsin_crab	TNLLVVNLAF	SDFCMMAFMM	PTMTSNCFAE	TWILG.....	..PFMCEVYG
	ET_Aprec	PNALIASLAL	GDLIYVVIDL	PINVFKLLAG	RWPFDH.NDF	GV.FLCKLFP
	ET_Bprec	PNILIASLAL	GDLHLHVIDI	PINVKLLAE	DWPFGE....	..MCKLVP
	ET_Cfrog	PNVLIASLAL	GDLFYILIAI	PIISISFWLS	TGH.....	..SEYIYQ
	galanin	TNLFILNLSI	ADLAYLLFCI	PFOATVYALP	TWVLGA....	..FICKFIH
60	NMB	PNIFISNLAA	GDLLLLLITCV	PVDASRYFFD	EWMEFGKVG..	..CKLIP
	GRP	PNLFISSLAL	GDLLLLLITCA	PVDASRYLAD	RWLFGRIG..	..CKLIP
	BRS3	PNIFITSLAF	GDLLLLLITCV	PVDATHYLAE	GWLFGRIG..	..CKVLS
	deltaOP	TNIYIFNLAL	ADALATSTLP	FQSAKYLMT	.WPFGE....	..LLCKAVL
	kappaOP	TNIYIFNLAL	ADALVTTTTP	FQSTVYLMNS	.WPFGE....	..VLCIKVI
	muOP	TNIYIFNLAL	ADALATSTLP	FQSVNYLMGT	.WPFGE....	..ILCKIVI
65	OPRX	TNIYIFNLAL	ADTLVLLTLP	FQGTDILLGF	.WPFGE....	..ALCKTVI
	CB1	SYHFIGSLAV	ADLLGSVIFV	YSFIDFHVH	RKD.....	..SRNVFLFKL
	CB2	SYLFIGSLAG	ADFLASVIFA	CSFVNHFVH	GVD.....	..SKAVFLKLI
	SSTR1	TNIYILNLAI	ADELLMLSVP	FLVTSTLLRH	.WPFGE....	..LLCRLVL

	SSTR2	TNIYILNLAI	ADELFLMLGLP	FLAMQVALVH	.WPF GK....	...AICRVVM
	SSTR3	TNVYILNLAL	ADELFLMLGLP	FLAAQNALS	.WPF GS....	...LMCRLVM
	SSTR4	TNIYLLNLAV	ADELFLMLSVP	FVASSAALRH	.WPF GS....	...VLCRAVL
5	SSTR5	TNIYILNLAV	ADVLYMLGLP	FLATQNAASF	.WPF GP....	...VLCRLVM
	IL8A	TDVYLLNLAL	ADLLFALTLP	IWAA..SKVN	GWIFGT....	...FLCKVVS
	IL8B	TDVYLLNLAL	ADLLFALTLP	IWAA..SKVN	GWIFGT....	...FLCKVVS
	AT1a	ASVFLNLAL	ADLCFLLTLP	LWAVYTAMEY	RWPFGN....	...YLCKIAS
	AT1brat	ASVFLNLAL	ADLCFLLTLP	LWAVYTAMEY	RWPFGN....	...HLCKIAS
10	AT2	SSIYIFNLAV	ADLLLLLATLP	LWATYYSYRY	DWLF GP....	...VMCKVFG
	BK1	AEIYLANLAA	SDLVFLGLP	FWAENIWNQF	NWPFGA....	...LLCRVIN
	BK2	AEIYLGNLAA	ADLILACGLP	FWAITISNNF	DWLFGE....	...TLCRVNV
	P2Y7	TALMVLNLAL	ADLAVLLTAP	FFLHFLAQGT	WSFGLA....	...GCRLCH
	P2Y6	SAVYTLNLAL	ADLLYACSLP	LLIYNYARGD	HWPF GD....	...LACRLVR
	P2Y5	TTYMLNLAI	SDLLFVFTLP	FRIYYFVVRN	.WPF GD....	...VLCKISV
15	P2Y4	TATYMFHLAL	SDTLVVSLLP	TLIYVYAAHN	HWPF GT....	...EICKFVR
	P2Y3chick	TTYMLNLAM	ADLLYVCSLP	LLIYNYTQKD	YWPFGD....	...FTCKFVR
	P2Y2	STTYMFHLAV	SDALYAASLP	LLVYYYARGD	HWPF ST....	...VLCKLVR
	P2Y1	ISVYMFNLAL	ADFLYVLTLP	ALIFYFYNKT	DWIF GD....	...AMCKLQR
20	THRprec	AVVYMLHLAT	ADVLFVSVLP	FKISYYFSGS	DWQFGS....	...ELCRFVT
	C5a	NAIWFLNLAV	ADFLSCLALP	ILFTSIVQHH	WPF GGA....	...ACSILP
	GP01mouse	MFLIGSLAL	ADLLAGLGLI	INFVFAYLLQ	....SE....	...ATKLVTI
	R334rat	MFLIGSLAL	ADLLAGLGLI	INFVFAYLLQ	....SE....	...ATKLVTI
	GP21mouse	MFLLVGSLAV	ADLLAGLGLV	LHFAADFCIG	....SP....	...EMSLMLV
25	GCRcmouse	TSLFIVNLAV	ADIMITLNT	PFTLVRVFN	TWVFGK....	...GMCHVSR
	TXKR	TNSFLVNLAF	ADAAMAALNA	LWNFIYALHG	EWYFGA....	...NYCRFQN
	G10Drat	LNLYILNMAV	ADLGIILSLP	VWMLVEMLEY	TWLWGS....	...FSCRFIH
	RDC1	.HCYILNLAI	ADLWVVLTP	VWVVSIVQHN	QWPMGE....	...LTCKVTH
	BLR1	..TFLFHLAV	ADLLLVFILP	FAVAEGSV..	GWVLGT....	...FLCKTVI
30	CL5	....LHLSV	ADLLFVITLP	FWAVDAVA..	NWYFGN....	...FLCKAVH
	LCR1	....LHLSV	ADLLFVITLP	FWAVDAVA..	NWYFGN....	...FLCKAVH
	EBI1	....LLNLAV	ADILFLLTLP	FWAYSAAK..	SWVFGV....	...HFCKLIF
	RBS1rat	....LLNLAL	SDLLFVATLP	FWTHYLIS..	HEGLHN....	...AMCKLTT
	EBI2	....STNLVI	SDILFTTALP	TRIAYYAMGF	DWRIGD....	...ALCRITA
35	GCRTchick	....MLNLAI	SDLLFVFTLP	FRIYYFVVR..	NWPFGD....	...VLCKISV
	APJ	..IFIASLAV	ADLTFVVTLP	LWATYTYRDI	DWPF GT....	...FFCKLSS
	RTArat	FSIYFLHLAS	ADGIYLF SKA	VIALLNMGTF	LGSFPD....	...YVRRVSR
	UHRrat	TNFLIGNLAL	SDVLMCAACV	PLTLAYAFEP	RGWVFG....	...GGLCHLVF
	FMRL1	NTICYLNLAL	ADFSFSAILP	FRMVSVAMRE	KWPFAS....	...FLCKLVH
40	FMRL2	TTICYLNLAL	ADFSFTATLP	FLIVSMAMGE	KWPF GW....	...FLCKLIH
	fMLP	TTISYLNLA	ADFCFTSTLP	FEMVRKAMGG	HWPF GW....	...FLCKFLF
	OLF1catfish	KYITVFNLA	SDLGGSSALI	PKLIDTFLF..	.....ENQV	ISYEACLANM
	OLF3catfish	KYIAVFNLA	SDLCGSSALI	PKLLDMLLF..	.....ENQS	ISYEACLSNM
	OLF8catfish	MCILIGLMAV	VDLSMPIFCV	PNMLLSFLF..	.....NWKG	ISLVGCLVQM
45	OLF32Acatfish	KYMGIFNLAL	SDFGETNVLI	PSLVKTLFF..	.....DSQY	ISYDACLANM
	OLF32Bcatfish	KYMGIFNLAL	SDFGETNALI	PSLVKTLFF..	.....DSQY	ISYDACLANM
	OLF32Ccatfish	KYMGIFNLAL	SDIGETNALI	PSLVKTLFF..	.....DSQY	ISYDACLTNM
	OLF32Dcatfish	KYMGIFNLAL	SDFGETNALI	PSLVKTLFF..	.....DSQY	ISYDACLANM
	OLF47catfish	KFLAVFNLA	VDISINSVII	PQMVPVVF..	.....NLNH	ISFESCF SQM
50	OLF202catfish	MYIIMLNLA	SDVLFSTTTL	PKIARYWF..	.....GDGS	ISFVGCFIQM
	OLFCOR1chicken	MYIFLQNL SF	TDAAYSTVIT	PKMLATFL..	.....EERT	ISYVGCILQY
	OLFCOR2chicken	MYIFLQNL SF	TDAAYSTVIT	PKMLATFL..	.....EERT	ISYVGCILQY
	OLFCOR3chicken	MYIFLQNL SF	TDAAYSTVIT	PKMLATFL..	.....EERT	ISYVGCILQY
	OLFCOR4chicken	MYIFLQNL SF	TDAAYSTVIT	PKMLATFL..	.....EERT	ISYVGCILQY
55	OLFCOR5chicken	MYIFLQNL SF	TDAAYSTVIT	PKMLATFL..	.....EERT	ISYVGCILQY
	OLFCOR6chicken	MYIFLQNL SF	TDAVYSTVIT	PKMLATFL..	.....EERT	ISYVGCILQY
	OLFdog	MYFLSNLSF	SDLCFSSVTM	PKLLQNMQ..	.....SQVPS	IPYAGCLTQM
	OLF07E	VYFFLANLSF	TDLFFVTNTI	PKMLVNLQ..	.....SHNKA	ISYAGCLTQL
	OLF07I	MYFLSNLSF	SDLCFSSVTI	PKLLQNMQ..	.....NQDPS	IPYADCLTQM
	OLF07J	MYFFLSMLST	SETVYTLVIL	PRMLSSLV..	.....GMSQP	MSLAGCATQM
60	OLFOR3mouse	MYFFLSNLSS	LDLAFTTSSV	PQMLKNLW..	.....GPDKT	ISYGGCVTQL
	OLFrat	MYFFLSLSF	VDLCYSTVIT	PKMLVNFL..	.....GKKNF	ITYSECMAQF
	OLFF3rat	MYFFLSNL SF	VDICFISTTV	PKMLVNIQ..	.....TQNNV	ITYAGCITQI
	OLFF5rat	MYFFLSNL SF	VDVCFSSSTTV	PKVLNHI..	.....LGSQA	ISFSGCLTQL
	OLFF6rat	MYFFLCNL SF	LEIWFTTACV	PKTLATF..	.....APRGV	ISLAGCATQM
65	OLFF12rat	MYFFLANLSF	VDICFTSTTI	PKMLVNIY..	.....TQSKS	ITYEDCISQM
	OLFI3rat	MYFLSNLSF	SDLCFSSVTM	PKLLQNM..	.....SQDTS	IPYGGCLAQ
	OLFI7rat	MYFFLANMSF	LEIWVTVITI	PKMLAGFIG..	..SKENHGQL	ISFEACMTQL
	OLFI8rat	MYFLSNLSF	SDLCFSSVTM	LKLLQNIQ..	.....SQVPS	ISYAGCLTQI



5      OLFI9rat   MYLFLSNLSF   ADLCFSSVTM   PKLLQNMQ..   .....SQVPS   IPYAGCLAQI  
       OLFI14rat   MYLFLSNLSF   SDLCFSSVTM   PKLLQNMQ..   .....SQVPS   ISYTGCLTQL  
       OLFI15rat   MYLFLSNLSF   SDLCFSSVTM   PKLLQNMQ..   .....SQVPS   IPFAGCLTQL  
       OLFOR17\_40   MYFFLGNSLV   LDVGCISVTV   PSMLSRLL..   .....SRKRA   VPCGACLTQL  
       GUST27rat   MYFFLSNLSF   VDICFISTTI   PKMLVNIH..   .....SQTkd   ISYIECLSQV  
       RPE   CHLLVLSLAL   ADSGISLNAL   VAATSSLLRR   WPYG.....   ..SDGCQAHG  
       HHRF1   GDVYFINLAA   ADLLFVCTLP   LWMQYLLDHN   SLA.....   ..SVPCTLLT  
       HHRF2   SDTYICNLAV   ADLLIVVGLP   FFLEYAKHHP   KLSR.....   ..EVVCSGLN  
       HHRF3   PTIYMTNLYS   TNFLTTLTVLP   FIVLSNQWLL   PAG.....   ..VASCKFLS  
 10      MCP-1A   TDIYLLNLAI   SDLLFLITLP   LWAHSAANEW   VFG.....   ..NAMCKLFT  
       MCP-1B   TDIYLLNLAI   SDLLFLITLP   LWAHSAANEW   VFG.....   ..NAMCKLFT  
       PPR1bovine   TDVYILNLAV   ADLFLFLITLP   FWAVNAVHGW   VLG.....   ..KIMCKVTS

15      The above sequences were obtained from a public database. Examples of TM3 and  
 TM5 transmembrane domain sequences are included in WO 94/05695, and are  
 incorporated by reference. Examples of TM4, TM6, and TM7 transmembrane domain  
 sequences can similarly be obtained from public sources

## 2. Synthesis of peptides

20      The peptides or fragments of GPCRs may be isolated from a natural  
 source, chemically synthesized or produced recombinantly, in order to provide  
 GPCR polypeptides which mimic, modulate or inhibit binding of ligands to G-protein  
 coupled receptors.

### a. Chemical synthesis of GPCR transmembrane peptides

25      Transmembrane peptides of the present invention are made using  
 well known peptide synthesis procedures, as described in e.g., Merrifield, *Science*  
 232: 341-347 (1986), Barany and Merrifield, *THE PEPTIDES*, Gross and Meienhofer,  
 eds. (N.Y., Academic Press), pp. 1-284 (1979); and Stewart and Young, *SOLID PHASE*  
*PEPTIDE SYNTHESIS* (Rockford, Ill., Pierce), 2d Ed. (1984), incorporated by reference  
 herein.

30      The peptides were synthesized by a flow-through solid phase peptide  
 synthesis on 432A Applied Biosystems Peptide Synthesizer utilizing Fmoc amino acid  
 derivatives. To overcome aggregation that frequently occurs during the synthesis of  
 hydrophobic peptides and leads to the blockage of the growing peptide chain,  
 FmocHmb derivatives of Ala, Val and Leu were introduced into the different  
 35      sequences; but not more than two derivatives of that type per peptide to prevent  
 steric hindrance during the synthesis. Coupling on the step after FmocHmb amino  
 acid was prolonged to 90 min, since this protection group causes slowing of the next  
 coupling step due to steric hindrance (T. Johnson, M. Quibell, *Tetrahedron Lett.*  
 35:463 (1994). The purity of the peptides was assessed by reverse phase HPLC and  
 40      the structures were confirmed by matrix-assisted laser-desorption mass spectrometry.



**b. Recombinant production of GPCR transmembrane peptides**

Nucleic acids that encode GPCR transmembrane peptides may be obtained by synthesizing, isolating or obtaining a nucleic acid sequence that encodes a GPCR protein, and subcloning a region of the sequence that encodes a desired transmembrane peptide.

**i. Chemical synthesis of oligonucleotides**

Oligonucleotides used in the present invention, including sequences that encode transmembrane peptides, are optionally chemically synthesized using the solid phase phosphoramidite triester method of Beaucage and Carruthers,

*Tetrahedron Lett.*, 22(20): 1859-1862 (1981) using an automated synthesizer as described in Needham-VanDevanter et al., *Nucleic Acids Res.*, 12: 6159-6168 (1984). The chemically synthesized oligonucleotides are then purified by native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson and Regnier, *J. Chrom.*, 255: 137-149 (1983). The sequence of the synthetic oligonucleotide is verified, for example by using the chemical degradation method of Maxam and Gilbert in Grossman, L. and Moldave, D., eds. Academic Press, New York, *Methods in Enzymology*, 65:499-560 (1980).

The DNA sequences of the present invention coding for GPCR transmembrane peptides protein can be modified (i.e., mutated) to prepare various mutations. Such mutations may be either degenerate, i.e., the mutation does not change the amino acid sequence encoded by the mutated codon, or non-degenerate, i.e., the mutation changes the amino acid sequence encoded by the mutated codon. These modified DNA sequences may be prepared, for example, by mutating known sequences so that the mutation results in the deletion, substitution, insertion, inversion or addition of one or more amino acids in the encoded polypeptide using various methods known in the art. For example, the methods of site-directed mutagenesis described in Taylor et al., *Nucl. Acids Res.* 13, 8749-8764 (1985) and Kunkel, *Proc. Natl. Acad. Sci. USA* 82, 482-492 (1985) may be employed. In addition, kits for site-directed mutagenesis may be purchased from commercial vendors. For example, a kit for performing site-directed mutagenesis may be purchased from Amersham Corp. (Arlington Heights, Ill.). Both degenerate and non-degenerate mutations may be advantageous in producing or using the polypeptides of the present invention. For example, these mutations may permit

higher levels of production, easier purification, or provide additional restriction endonuclease recognition sites. All such modified DNAs (and the encoded polypeptide molecules) are included within the scope of the present invention.

**ii. Recombinant isolation of GPCR transmembrane peptide-encoding nucleic acids**

Nucleic acids that encode GPCR can be isolated from genomic or cDNA libraries, subcloning the library into expression vectors, labelling probes, DNA hybridization, and the like, as described in Sambrook, et al., MOLECULAR CLONING - A LABORATORY MANUAL (2nd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989. This manual is hereinafter referred to as "Sambrook, et al.", and is incorporated herein by reference.

Various methods of amplifying target sequences, such as the polymerase chain reaction (PCR), can also be used to prepare DNA encoding GPCR transmembrane peptides or a peptide fragment thereof. In PCR techniques, oligonucleotide primers complementary to the two 3' borders of the DNA region to be amplified are synthesized. The polymerase chain reaction is then carried out using the two primers. See PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS. (Innis, M, Gelfand, D., Sninsky, J. and White, T., eds.), Academic Press, San Diego (1990). Primers can be selected to amplify the entire regions encoding a full-length GPCR transmembrane peptides or to amplify smaller DNA segments as desired. Once selected sequences are PCR-amplified, oligonucleotide probes can be prepared from sequence obtained. These probes can then be used to isolate DNA's encoding GPCR transmembrane peptides or a peptide fragment thereof.

**iii. Recombinant expression of transmembrane peptide-encoding nucleic acids**

Once a nucleic acid encoding a GPCR transmembrane peptides or a peptide fragment thereof is isolated and cloned, the nucleic acid is expressed in a variety of recombinantly engineered cells to ascertain that the isolated nucleic acid indeed encodes the desired GPCR transmembrane peptides or a peptide fragment thereof. The expression of natural or synthetic nucleic acids is typically achieved by operably linking a nucleic acid of interest to a promoter (which is either constitutive or inducible), incorporating the construct into an expression vector, and introducing

the vector into a suitable host cell. Typical vectors contain transcription and translation terminators, transcription and translation initiation sequences, and promoters useful for regulation of the expression of the particular nucleic acid. The vectors optionally comprise generic expression cassettes containing at least one independent terminator sequence, sequences permitting replication of the cassette in eukaryotes, or prokaryotes, or both, (e.g., shuttle vectors) and selection markers for both prokaryotic and eukaryotic systems. Vectors are suitable for replication and integration in prokaryotes, eukaryotes, or preferably both. See, Gilman and Smith (1979), *Gene*, 8: 81-97; Roberts et al. (1987), *Nature*, 328:731-734; Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* 152, Academic Press, Inc., San Diego, CA (Berger); Sambrook et al. (1989), *MOLECULAR CLONING - A LABORATORY MANUAL* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, N.Y., (Sambrook); and F.M. Ausubel et al., *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel). Product information from manufacturers of biological reagents and experimental equipment also provide information useful in known biological methods. Such manufacturers include the SIGMA chemical company (Saint Louis, MO), R&D systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem. Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), and Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill.

The nucleic acids (e.g., coding sequences, promoters and vectors) used in the present method can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries, or prepared by synthetic methods. Synthetic nucleic acids can be prepared by a variety of solution or solid phase methods. Detailed descriptions of the procedures for solid phase synthesis of nucleic acids by phosphite-triester, phosphotriester, and H-phosphonate chemistries are widely available. See, for example, Itakura, U.S. Pat. No. 4,401,796; Caruthers, et al., U.S. Pat. Nos. 4,458,066 and 4,500,707; Beaucage, et al., (1981) *Tetrahedron Lett.*, 22:1859-1862; Matteucci, (1981) et al., *J. Am. Chem. Soc.*, 103:3185-3191;

Caruthers, et al., (1982) *Genetic Engineering*, 4:1-17; Jones, chapter 2, Atkinson, et al., chapter 3, and Sproat, et al., chapter 4, in OLIGONUCLEOTIDE SYNTHESIS: A PRACTICAL APPROACH, Gait (ed.), IRL Press, Washington D.C. (1984); Froehler, et al., (1986) *Tetrahedron Lett.*, 27:469-472; Froehler, et al., (1986) *Nucleic Acids Res.*, 14:5399-5407; Sinha, et al. (1983) *Tetrahedron Lett.*, 24:5843-5846; and Sinha, et al., (1984) *Nucl. Acids Res.*, 12:4539-4557, which are incorporated herein by reference.

### 3. Derivatized Peptides and Peptidomimetics

The design of chemically modified peptides and peptide mimics which are resistant to degradation by proteolytic enzymes or have improved solubility or binding properties is well known.

Modified amino acids or chemical derivatives of GPCRs peptides according to the present invention may contain additional chemical moieties or modified amino acids not normally a part of the protein. Covalent modifications of the peptide are thus included within the scope of the present invention. Such modifications may be introduced into a GPCR polypeptide by reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. The following examples of chemical derivatives are provided by way of illustration and not by way of limitation.

The design of peptide mimics which are resistant to degradation by proteolytic enzymes is well known, both for hormone agonist/antagonist and for enzyme inhibitor design. See e.g., Sawyer, in STRUCTURE-BASED DRUG DESIGN, P. Verapandia, Ed., NY 1997; U.S. Patent No. 5,552,534; and U.S. Patent No. 5,550,251, all of which are incorporated by reference.

Historically, the major focus of peptidomimetic design has evolved from receptor-targeted drug discovery research and has not been directly impacted by an experimentally-determined three-dimensional structure of the target protein. Nevertheless, a hierarchical approach of peptide→peptidomimetic molecular design and chemical modification has evolved over the past two decades, based on systematic transformation of a peptide ligand and iterative analysis of the structure-activity and structure-conformation relationships of "second generation" analogs. Such work has typically integrated biophysical techniques (x-ray crystallography

and/or NMR spectroscopy) and computer-assisted molecular modeling with biological testing to advance peptidomimetic drug design.

The three-dimensional structural properties of peptides are defined in terms of torsion angles ( $\Psi$ ,  $\phi$ ,  $\omega$ ,  $\chi$ ) between the backbone amine nitrogen ( $N^\alpha$ ), backbone carbonyl carbon ( $C^1$ ), backbone methionine carbon ( $C^\alpha$ ), and side chain hydrocarbon functionalization (e.g.,  $C^\beta$ ,  $C^\gamma$ ,  $C^\delta$ ,  $C^\epsilon$  of Lys) derived from the amino acid sequence. A Ramachandran plot ( $\Psi$  versus  $\phi$ ) may define the preferred combinations of torsion angles for ordered secondary structures (conformations), such as  $\alpha$  helix,  $\beta$  turn,  $\gamma$  turn, or  $\beta$  sheet. Molecular flexibility is directly related to covalent and/or noncovalent bonding interactions within a particular peptide. Even modest chemical modifications by  $N^\alpha$ -methyl,  $C^\alpha$ -methyl or  $C^\beta$ -methyl can have significant consequences on the resultant conformation.

The  $N^\alpha$ - $C^\alpha$ - $C'$  scaffold may be transformed by introduction of olefin substitution (e.g.,  $C^\alpha$ - $C^\beta \rightarrow C=C$  or dehydroamino acid or insertion (e.g.,  $C^\alpha$ - $C' \rightarrow C^\alpha$ - $C=C-C'$  or vinylogous amino acid. Also the  $C^\beta$  carbon may be substituted to advance the design of so-called "chimeric" amino acids. Finally, with respect to N-substituted amides it is also noteworthy to mention the intriguing approach of replacing the traditional peptide scaffold by achiral N-substituted glycine building blocks. Overall, such  $N^\alpha$ - $C^\alpha$ - $C$  scaffold or  $C^\alpha$ - $C^\beta$  side chain modifications expand peptide-based molecular diversity (i.e., so-called "peptoid" libraries) as well as extend our 3-D structural knowledge of traditional  $\phi$ - $\Psi$ - $\chi$  space.

In one approach, such as disclosed by Sherman and Spatola, *J. Am. Chem. Soc.* 112: 433 (1990), one or more amide bonds are replaced in an essentially isosteric manner by a variety of chemical functional groups. For example, any amide linkage in any of the GPCR polypeptides can be replaced by a ketomethylene moiety, e.g.  $-(C(=O)-CH_2-)$  for  $-(C(=O)-NH-)$ . A few of the known amide bond replacements include: aminomethylene or  $\Psi[CH_2NH]$ ; ketomethylene or  $\Psi[COCH_2]$ ; ethylene or  $\Psi[CH_2CH_2]$ ; olefin or  $\Psi[CH=CH]$ ; ether or  $\Psi[CH_2O]$ ; thioether or  $\Psi[CH_2S]$ ; tetrazole or  $\Psi[CN_4]$ ; thiazole or  $\Psi[thz]$ ; retroamide or  $\Psi[NHCO]$ ; thioamide or  $\Psi[CSNH]$ ; and ester or  $\Psi[CO_2]$ . These amide bond surrogates alter conformational and H-bonding properties that may be requisite for peptide molecular recognition and/or biological activity at receptor targets. Furthermore, such backbone replacements can impart metabolic stability towards peptidase cleavage

relative to the parent peptide. The discovery of yet other nonhydrolyzable amide bond isostere has particularly impacted the design of protease inhibitors, and these include: hydroxymethylene or  $\Psi[\text{CH}(\text{OH})]$ ; hydroxyethylene or  $\Psi[\text{CH}(\text{OH})\text{CH}_2]$  and  $\Psi[\text{CH}_2\text{CH}(\text{OH})]$ ; dihydroxyethylene or  $(\Psi[\text{CH}(\text{OH})\text{CH}(\text{OH})])$ , hydroxyethylamine or  $\Psi[\text{CH}(\text{OH})\text{CH}_2\text{N}]$ , dihydroxyethylene and  $\text{C}_2$ -symmetric hydroxymethylene. Such backbone modifications have been extremely effective, as they may represent transition state mimics or bioisosteres of the hypothetical tetrahedral intermediate (e.g.,  $\Psi[\text{C}(\text{OH})_2\text{NH}]$ ) for this class of proteolytic enzymes. Such derivatives are expected to have the property of increased stability to degradation by enzymes, and therefore possess advantages for the formulation of compounds which may have increased *in vivo* half lives, as administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

Both peptide backbone and side chain modifications may provide prototypic leads for the design of secondary structure mimicry, as typically suggested by the fact that substitution of D-amino acids,  $\text{N}^\alpha$ -Me-amino acids,  $\text{C}_\alpha$ -Me amino acids, and/or dehydroamino acids within a peptide lead may induce or stabilize regiospecific  $\beta$ -turn,  $\gamma$ -turn,  $\beta$ -sheet, or  $\alpha$ -helix conformations. To date, a variety of secondary structure mimetics have been designed and incorporated in peptides or peptidomimetics. The  $\beta$ -turn has been of particular interest to the area of receptor-targeted peptidomimetic drug discovery. This secondary structural motif exists within a tetrapeptide sequence in which the first and fourth  $\text{C}_\alpha$  atoms are  $\leq 7 \text{ \AA}$  separated, and they are further characterized as to occur in a nonhelical region of the peptide sequence and to possess a ten-membered intramolecular H-bond between the  $i$  and  $i+4$  amino acid residues. One of the initial approaches of significance to the design of  $\beta$ -turn mimetics was the monocyclic dipeptide-based template which employs side chain to backbone constraint at the  $i+1$  and  $i+2$  sites. Over the past decade a variety of other monocyclic or bicyclic templates have been developed as  $\beta$ -turn mimetics. Monocyclic  $\beta$ -turn mimetic has been described that illustrate the potential opportunity to design scaffolds that may incorporate each of the side chains ( $i$ ,  $i+1$ ,  $i+2$  and  $i+3$  positions), as well as five of the eight NH or C=O functionalities, within the parent tetrapeptide sequence, tetrapeptide sequence modeled in type I-IV  $\beta$ -turn conformations. Similarly, a benzodiazepine template has shown utility as a  $\beta$ -turn mimetic scaffold which also may be multisubstituted to simulate side chain

functionalization,, particularly at the *i* and *i* + 3 positions of the corresponding tetrapeptide sequence modeled in type I-VI  $\beta$ -turn conformations. A recently reported  $\gamma$ -turn mimetic, illustrates an innovative approach to incorporate a retroamide surrogate between the *i* and *i*→1 amino acid residues with an ethylene bridge between the N<sup>1</sup> (i.e., nitrogen replacing the carbonyl C') and N atoms of the *i* and *i* + 2 positions, and this template allows the possibility for all three side chains of the parent tripeptide sequence. Finally, the design of a  $\beta$ -sheet mimetic provides an attractive template to constrain the backbone of a peptide to that simulating an extended conformation. The  $\beta$ -sheet is of particular interest to the area of protease-targeted peptidomimetic drug discovery.

Aromatic amino acids may be replaced with D- or L-naphthylalanine, D- or L-phenylglycine, D- or L-2-thieneylalanine, D- or L-1-, 2-, 3- or 4-pyreneylalanine, D- or L-3-thieneylalanine, D- or L-(2-pyridinyl)-alanine, D- or L-(3-pyridinyl)-alanine, D- or L-(2-pyrazinyl)-alanine, D- or L-(4-isopropyl)-phenylglycine, D-(trifluoromethyl)-phenylglycine, D-(trifluoromethyl)-phenylalanine, D-p-fluorophenylalanine, D- or L-p-biphenylphenylalanine, D- or L-p-methoxybiphenylphenylalanine, D- or L-2-indole(alkyl)alanines, and D- or L-alkylainines where alkyl may be substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, isopropyl, iso-butyl, sec-isotyl, iso-pentyl, non-acidic amino acids, of C<sub>1</sub>-C<sub>20</sub>.

Acidic amino acids can be substituted with non-carboxylate amino acids while maintaining a negative charge, and derivatives or analogs thereof, such as the non-limiting examples of (phosphono)alanine, glycine, leucine, isoleucine, threonine, or serine; or sulfated (e.g., -SO<sub>3</sub>H) threonine, serine, tyrosine.

Other substitutions may include unnatural hydroxylated amino acids made by combining "alkyl" (as defined and exemplified herein) with any natural amino acid. Basic amino acids may be substituted with alkyl groups at any position of the naturally occurring amino acids lysine, arginine, ornithine, citrulline, or (guanidino)-acetic acid, or other (guanidino)alkyl-acetic acids, where "alkyl" is defined as above. Nitrile derivatives (e.g., containing the CN-moiety in place of COOH) may also be substituted for asparagine or glutamine, and methionine sulfoxide may be substituted for methionine. Methods of preparation of such peptide derivatives are well known to one skilled in the art.

In addition, any amino acid of said peptides can be replaced by the same amino acid but of the opposite chirality. Thus, any amino acid naturally occurring in the L-configuration (which may also be referred to as the R or S, depending upon the structure of the chemical entity) may be replaced with an amino acid of the same chemical structural type, but of the opposite caroled, generally referred to as the D- amino acid but which can additionally be referred to as the R- or the S-, depending upon its composition and chemical configuration. Such derivatives have the property of greatly increased stability co degradation by enzymes, and therefore are advantageous in the formulation of compounds which may have longer *in vivo* half lives, when administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

Additional amino acid modifications of amino acids of GPCR polypeptides of to the present invention may include the following: Cysteiny residues may be reacted with alpha-haloacetates (and corresponding amines), such as 2-chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteiny residues may also be derivatized by reaction with compounds such as bromotrifluoroacetone, alpha-bromo-beta-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues may be derivatized by reaction with compounds such as diethylprocarbonate e.g., at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain, and para-bromophenacyl bromide may also be used; e.g., where the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysiny and amino terminal residues may be reacted with compounds such as succinic or other carboxylic acid anhydrides. Derivatization with these agents is expected to have the effect of reversing the charge of the lysiny residues. Other suitable reagents for derivatizing alpha-amino-containing residues include compounds such as imidoesters/e.g., as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.



Arginyl residues may be modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin according to known method steps. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

Tyrosyl residues may be modified by reaction with aromatic diazonium compounds or tetranitromethane. N-acetylimidazol and tetranitromethane may be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides ( $R'-N-C-N-R'$ ) such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore aspartyl and glutamyl residues may be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues may be frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues may be deamidated under mildly acidic conditions. Either form of these residues falls within the scope of the present invention.

Derivatization with bifunctional agents is useful for cross-linking the peptide to certain chemical moieties. Commonly used cross-linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 (which are herein incorporated entirely by reference), may be employed for protein immobilization.

Other modifications of GPCR polypeptides of the present invention may include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups

of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, *Proteins: Structure and Molecule Properties*, W. H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, methylation of main chain amide residues (or substitution with N-methyl amino acids) and, in some instances, amidation of the C-terminal carboxyl groups, according to known method steps.

Such derivatized moieties may improve the solubility, absorption, permeability across the blood brain barrier biological half life, and the like. Such moieties or modifications of GPCR polypeptides may alternatively eliminate or attenuate any possible undesirable side effect of the protein and the like. Moieties capable of mediating such effects are disclosed, for example, in Remington's *Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, Pa. (1980).

Such chemical derivatives of GPCR polypeptides also may provide attachment to solid supports, including but not limited to, agarose, cellulose, hollow fibers, or other polymeric carbohydrates such as agarose, cellulose, such as for purification, generation of antibodies or cloning; or to provide altered physical properties, such as resistance to enzymatic degradation or increased binding affinity or modulation for GPCRs, which is desired for therapeutic compositions comprising GPCR polypeptides, antibodies thereto or fragments thereof. Such peptide derivatives are well-known in the art, as well as method steps for making such derivatives using carbodiimides active esters of N-hydroxy succinimide, or mixed anhydrides, as non-limiting examples.

Variation upon the sequences of GPCR polypeptides of the present invention may also include: the addition of one or more (e.g., two, three, four, or five) lysine, arginine or other basic residues or one, or more (e.g., two, three, four, or five) glutamate or aspartate or other acidic residues at one end of the peptide, where "acidic" and "basic" are as defined herein. Negative charges can also be introduced by the addition of carboxyl, phosphate, borate, sulfonate or sulfate groups. Such modifications may increase the alpha-helical content of the peptide by the "helix dipole effect". They also can provide enhanced aqueous solubility of the peptide, and allow the correct insertion of peptides into a membrane structure.

In another approach, a variety of uncoded or modified amino acids such as D-amino acids and N-methyl amino acids have been used to modify

mammalian peptides. Alternatively, a presumed bioactive conformation has been stabilized by a covalent modification, such as cyclization or by incorporation of gamma-lactam or other types of bridges. See, e.g., Veber and Hirschmann, *et al.*, *Proc. Natl. Acad. Sci. USA*, 1978 75 2636 and Thorsett, *et al.*, *Biochem Biophys. Res. Comm.*, 1983 111 166. The primary purpose of such manipulations has not been to avoid metabolism or to enhance oral bioavailability but rather to constrain a bioactive conformation to enhance potency or to induce greater specificity for a receptor subtype.

The above examples of peptide scaffold- or nonpeptide template-based peptidomimetic agonists or antagonists illustrate various strategies to elaborate bioactive conformation and/or pharmacophore models of peptide ligands at their receptors. In many cases, receptor subtype selectivity has also been achieved by systematic structural modifications of prototypic leads of peptidomimetics. Thus, although the 3D structures of GPCRs remains elusive (except for models constructed from homology-based low-resolution 3D structures of bacteriorhodopsin or rhodopsin, see below) the development of pharmacophore models using the hierarchical approach in peptide → peptidomimetic structure-based drug design is promising.

#### 4. Purification of GPCR transmembrane peptides

The polypeptides of this invention may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate, column chromatography, immunopurification methods, and others. See, for instance, R. Scopes, *Protein Purification: Principles and Practice*, Springer-Verlag: New York (1982), incorporated by reference. For example, the GPCR transmembrane peptides proteins and polypeptides produced by recombinant DNA technology are purified by a combination of cell lysis (e.g., sonication) and affinity chromatography or immunoprecipitation with a specific antibody to GPCR transmembrane peptides or a peptide fragment thereof. For fusion products, subsequent digestion of the fusion protein with an appropriate proteolytic enzyme releases the desired polypeptide. The proteins may then be further purified by standard protein chemistry techniques. A purified protein preferably exhibits a single band on an electrophoretic gel. Those of skill are reminded that the methods should take into account the hydrophobic nature of the peptides.

## 5. Detection of GPCR transmembrane peptide gene products

GPCR transmembrane peptides or a peptide fragment thereof to may be detected or quantified by a variety of methods. Preferred methods involve the use of specific antibodies.

### 5 a. Detection of GPCR transmembrane peptides by Immunoassay

#### i. Antibody Production

Methods of producing polyclonal and monoclonal antibodies are known to those of skill in the art. See, e.g., Coligan (1991), CURRENT PROTOCOLS IN IMMUNOLOGY, Wiley/Greene, NY; and Harlow and Lane (1989), ANTIBODIES: A LABORATORY MANUAL, Cold Spring Harbor Press, NY; Stites et al. (eds.) BASIC AND CLINICAL IMMUNOLOGY (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Goding (1986), MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975), *Nature*, 256:495-497. Such techniques include antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors. See, Huse et al. (1989), *Science*, 246:1275-1281; and Ward et al. (1989) *Nature*, 341:544-546. For example, in order to produce antisera for use in an immunoassay, a polypeptide is isolated as described herein. For example, recombinant protein is produced in a transformed cell line. An inbred strain of mice or rabbits is immunized with the peptide using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol. Alternatively, a synthetic peptide derived from the sequences disclosed herein and conjugated to a carrier protein can be used as an immunogen.

A number of immunogens may be used to produce antibodies specifically reactive with GPCR transmembrane peptides or a peptide fragment thereof. Recombinant protein is the preferred immunogen for the production of monoclonal or polyclonal antibodies. Naturally occurring protein may also be used either in pure or impure form. Synthetic peptides made using the GPCR transmembrane peptides or a peptide fragment thereof sequences described herein may also be used as an immunogen for the production of antibodies to the protein. Recombinant protein can be expressed in eukaryotic or prokaryotic cells as described above, and purified as generally described above. The product is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal

antibodies may be generated, for subsequent use in immunoassays to measure the protein.

Methods of production of polyclonal antibodies are known to those of skill in the art. In brief, an immunogen, preferably a purified protein such as GPCR transmembrane peptides or a peptide fragment thereof is mixed with an adjuvant and injected into an animal of choice (e.g., a mouse, rat, rabbit, pig, goat, cow, horse, chicken, etc.) at intervals of 1-4 weeks. The immunogen may be conjugated to a carrier protein can be used an immunogen. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the GPCR transmembrane peptides or a peptide fragment thereof. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired. (See Harlow and Lane, *supra*).

Polyclonal sera are collected and titered against the immunogen protein in an immunoassay, for example, a solid phase immunoassay with the immunogen immobilized on a solid support. Polyclonal antisera with a titer of  $10^4$  or greater are selected and tested for their cross reactivity against non-GPCR transmembrane peptides or even GPCR transmembrane peptides from other cell types or species or a peptide fragment thereof, using a competitive binding immunoassay (see, e.g., Harlow and Lane, *supra*, at pages 570-573). Specific monoclonal and polyclonal antibodies and antisera will usually bind with a  $K_D$  of at least about 0.1 mM, more usually at least about 1  $\mu$ M, preferably at least about .1  $\mu$ M or better, and most preferably, .01  $\mu$ M or better.

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (See, Kohler and Milstein, *Eur. J. Immunol.* 6:511-519 (1976), incorporated herein by reference). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells may be enhanced by various

techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined by Huse, et al. (1989) *Science* 246:1275-1281.

## ii. Immunoassays

A particular protein can be measured by a variety of immunoassay methods. For a review of immunological and immunoassay procedures in general, see BASIC AND CLINICAL IMMUNOLOGY, 7th Edition (D. Stites and A. Terr, eds.) 1991. Moreover, the immunoassays of the present invention can be performed in any of several configurations, which are reviewed extensively in ENZYME IMMUNOASSAY, E.T. Maggio, ed., CRC Press, Boca Raton, Florida (1980); "Practice and Theory of Enzyme Immunoassays," P. Tijssen, in LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY, Elsevier Science Publishers B.V. Amsterdam (1985); and, Harlow and Lane, ANTIBODIES, A LABORATORY MANUAL, *supra*, each of which is incorporated herein by reference.

Immunoassays to GPCR transmembrane peptides, peptidomimetics or subfragments thereof may use a polyclonal antiserum raised against a peptide or peptidomimetic of the invention. This antiserum is selected to have low cross-reactivity against other (other non-GPCR transmembrane peptides or other GPCR transmembrane peptides) peptides and any such cross-reactivity is removed by immunoabsorption prior to use in the immunoassay.

Immunoassays in the competitive binding format can be used for the crossreactivity determinations. For example, a reference peptide antigen of the invention can be immobilized to a solid support. The ability of other molecules (other GPCR transmembrane peptides, or non-GPCR transmembrane peptides, or unknowns) to compete with the binding of antisera which recognize the immobilized reference peptide antigen is measured. The ability of such molecules to compete with the binding of an antiserum or antibody to the immobilized reference peptide is compared to a standard molecule, such as the reference peptide antigen itself. The percent crossreactivity is calculated, using standard calculations. Antisera with less than 10% crossreactivity to crossreacting molecules are selected and pooled. Any

cross-reacting antibodies are optionally removed from the pooled antisera by immunoabsorption with cross-reacting molecules.

The immunoabsorbed and pooled antisera are then used in a competitive binding immunoassay to compare the binding of a second protein to that of the reference peptide antigen. In order to make this comparison, the two molecules are each assayed at a wide range of concentrations and the amount of each molecule required to inhibit 50% of the binding of the antisera to the immobilized reference peptide antigen is determined. If the amount of the second protein required is less than 10 times the amount of the reference peptide used to make the antibody, then the second protein is said to specifically bind to an antibody generated to the reference peptide antigen.

The presence of a desired polypeptide (including peptide, translation product, or enzymatic digestion product) in a sample may be detected and quantified using Western blot analysis. The technique generally comprises separating sample products by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with labeling antibodies that specifically bind to the analyte protein. The labeling antibodies specifically bind to analyte on the solid support. These antibodies are directly labeled, or alternatively are subsequently detected using labeling agents such as antibodies (e.g., labeled sheep anti-mouse antibodies where the antibody to an analyte is a murine antibody) that specifically bind to the labeling antibody.

#### **6. Detection of GPCR transmembrane peptide sequences, peptides and peptidomimetics that optimally inhibit GPCR biological properties and functions**

Peptides or peptide variants of the invention that modulate biological activity of GPCRs are generally identified as follows. Peptide sequences are selected from the transmembrane domains of the GPCR to be targeted. The transmembrane domains are readily ascertained by the application of computer models to known sequences. Computer modeling and comparison with known transmembrane peptide sequences are also used to define the orientation of the peptide sequence in the membrane, thus allowing the determination of the end of the peptide sequence that is towards the extracellular aspect of the plasma membrane. The selection of a

preferred transmembrane domain to be targeted is largely empirical. We have found that peptides derived from transmembrane domain 2 are particularly effective inhibitors of GPCR function. Alternatively, peptide sequences selected from transmembrane domain 4 have also been effective antagonists.

5           Upon selection of a peptide sequence, a reference transmembrane sequence is synthesized and systematically modified to identify variants (or analogs) that have improved properties. The modifications introduce a negative charge at the extracellular end of the peptide sequence. Negative charges may be added in the form of acidic amino acid residues such as Asp or Glu. The number of acidic  
10 residues that is added is typically from 1 to 3 depending upon the hydrophobicity of the peptide sequence and the subsequent necessity to increase the solubility of the peptide. Further, preferable peptides have a neutral charge at the end of the peptide that is oriented towards the intracellular aspect of the plasma membrane. Thus, the overall hydrophobic nature of such a transmembrane peptide will result in insertion  
15 into a membrane and the negative charge at the extracellular end will result in the peptide having the same orientation as the transmembrane GPCR domain from which it is derived. Insertion into the membrane may be tested by fluorescent microscopy of labeled peptide analogs using methodology known to those of skill in the art as illustrated in Example 3 herein.

20           The ability of the peptide or peptide variants to modulate activity of the targeted GPCR is generally determined by testing the ability of the peptide to inhibit activation that is induced by a natural ligand of the targeted GPCR. Activation of most GPCRs results in an increase in cAMP or the release of intracellular calcium. Thus, if activation of the target GPCR increases cAMP, the inhibitory activity of the  
25 peptide is determined by measuring cAMP levels using methods known to those in the art (see e.g., C. Nordstedt and B.B. Fredholm *Anal. Chem.* 189: 231-234 (1990). Similarly, if activation of the target GPCR releases intracellular calcium, the inhibitory activity of the peptide is determined by measuring the intracellular calcium levels as illustrated in the examples below.

30           Peptides may be tested for other properties including the following:

- enhanced ability to modulate GPCR activity;
- increased resistance to proteolysis;
- improved solubility;



- longer or shorter half-life, particularly in culture medium or a biological fluid such as plasma or whole blood;
- improved ability to insert into a membrane compartment, especially in a particular orientation, by means known in the art.

5 Variant peptides may also be synthesized having any one or more of the following modifications:

- conservative or non-conservative substitution of any of the amino acid residues;
- deletion or addition of residues at any position;
- 10 - chemical modification at any residue;
- peptidomimetic analogs of the reference peptide.

Variant peptides can be rationally designed and/or screened for using high throughput screening methodologies applied to combinatorial libraries. Methods of generating combinatorial libraries and screening such libraries using high-throughput  
15 methods are well known to those of skill in the art (see, e.g., Baum, *C&EN* (Feb. 7, 1994): 20-26 and references cited therein).

These variant peptides are also tested for the any of the above-listed properties. In general, a variant peptide is considered to have improved properties relative to the reference peptide if a given measurable property or parameter  
20 associated with the peptide has a value that is at least 10%, preferably at least 30%, more preferably at least 75%, and most preferably at least 95% better than the value for the reference peptide.

The relative ability of the modified peptides (as compared to the reference peptide) to modulate a GPCR biological activity is tested as follows. A cell  
25 line that expresses a GPCR and exhibits a GPCR-mediated biological activity is exposed to either the reference or the modified peptide under identical conditions, and the biological property of the GPCR is measured in the absence or presence of either peptide. Examples of cell lines, GPCRs expressed by the cell line, and GPCR-regulated properties measured include the following:

- 30 - any cell that stably expresses CXCR4, especially attached cells, , including cells that are genetically engineered to express CXCR4, including HeLa cells; CXCR4; stroma cell derived factor 1 -induced calcium flux;

- any cell that stably expresses CXCR4, especially attached cells, including cells that are genetically engineered to express CXCR4, including CM cells; CXCR4; HIV-1 infection;
- 5    - any cell that stably expresses CCKAR, especially attached cells, including cells that are genetically engineered to express CCKAR, such as CHO cells; CCKAR; cholecystokinin-induced calcium release;
- any cell that stably expresses human CCR5, especially attached cells, including cells that are genetically engineered to express CCR5, including HEK cells; CCR5; RANTES induced calcium release.

10           The inhibitory activity is measured by exposing GPCR-expressing cells to a range of concentrations of a test antagonist, and measuring a biological property or activity associated with that GPCR. The test concentrations can range from 1 nanomolar to 100 micromolar, depending on peptide solubility and affinity. Initial screening is performed using 10-fold dilutions, such as 50, 5, 0.5, 0.05 micromolar.

15   Then, the lowest active concentration is lowered in decrements of 10% to determine the lowest effective concentration. The property measured can be binding to a ligand (for example, binding of cholecystokinin octapeptide to CCKAR), or production of a measurable metabolic response (e.g., altered ion flux or translocation, altered phosphorylation, altered protein synthesis or degradation,

20   altered cellular morphology, altered secretion, altered production of particular components such as soluble inositol phosphates, binding of a virus and subsequent infection, tumor growth, chemotaxis, mitogenic response, cell growth activation, secretion, muscle contraction, vasopressing and vasodepressing activity, synaptic transmission, and release of intracellular calcium, etc.)

25           The following GPCRs have been reported to play a role in HIV infection:

STRL33	U.S. Provisional Application No. 60/042,880;
CCRR5	U.S. Patent Application No. 60/042,880;
CCR8:	U.S. Provisional Application No. 60/054,094;
30   CCR2	<i>Proc. Natl. Acad. Sci. USA</i> : 2752-2756 (1994) <i>J. Biol. Chem.</i> 270: 29671-29675 (1995)
CCR3:	<i>J. Biol. Chem.</i> 270: 16491-16494 (1995)
CX3CR1:	<i>DNA Cell Biol.</i> 14: 673-680 (1995)

The following is a list of transmembrane peptides that have GPCR antagonist properties:

From the GPCR CXCR4

F-2-2: LLFVITLPFWAVDAVANWYFGNDD

5 F-2-5: LLFVITLPFWAVDAVANDD-OH

F-4-2: VYVGWIPALLLTIPDFIFANDD-OH

F-6-1: VILILAFFACWLPYYIGISID-OH

F-7-3: DDEALAFFHCCLNPILYAFL-NH<sub>2</sub>

F-7-4: DDSITEALAFFHCCLNPILYAFL-NH<sub>2</sub>

10

From the GPCR CCR5

CCR5-TM-2-2: LFFL LTVPFWAHYAAAQWDFGDD

CCR5-TM-4-1: FGVVTSVITWVAVFASLPGIIFTSSDD

CCR5-TM-6-1: LIFTIMIVYFLFWAPYNIVLLLNTFQED

15 CCR5-TM-7-1: DDQAMQVTETLGMTHCCINPIIYAFV

From the GPCR CCR2

CCR2-TM-2-1: IYLLNLAISDLLFLITLPLWADD-OH

CCR2-TM-2-2: LLFLITLPLWAH SAANEWVFGNDD-OH

20 CCR2-TM-4-1: FGVVTSVITWLVAVF ASVPGIIFTDD

CCR2-TM-6-1: VIFTIMIVYFLFWTPYN IVILLNTFQED

CCR2-TM-7-1: DDATQVT ETLGMTHCCINPIIYAFV

From the GPCR CCR3

25 CCR3-TM-2-1: LLFLVTLPFW IHYVRGHNWVFGDDD

CCR3-TM-4-1: FGVITSIVTWGLAVLAALPEFI FYETED

CCR3-TM-6-1: IFVIMAVFFI FWTPYNVAILLSSYQSDD

CCR3-TM-7-1: DDLVMLVTEVIAYSHCCMNPVIYAFV

30 From the GPCR CCKAR

CCKAR-TM-1-6: DDEWQSALQILLYSIIFLLSVLGNTLVITV

CCKAR-TN-2-1: FLLSLAVSDLMLCLFCMPFNLP

CCKAR-TM-2-2: FLLSLAVSDLMLCLFCM PFNLIDD

CCKAR-TM-6-4: IVVLFFLCWMPIFSANAWRAYDTVDD

## 7. Treatment embodiments

The compositions containing the present GPCR transmembrane peptides, or a cocktail thereof (i.e., with other molecules, including other peptides of the invention), can be administered for therapeutic treatments. The molecules of the present invention are used to protect a patient from pathologies associated with GPCR, by modulating the biological activities associated with the GPCR.

"Protection" from infection or disease as used herein is intended to encompass "prevention" or "treatment." "Treatment" involves administration of the protective composition to a patient exhibiting symptoms of a GPCR-associated pathology (for example, HIV-1 infection), so as to reduce or suppress the symptoms of the pathology. Other examples of GPCR-associated conditions that may be treated with the peptides of the invention include:

- cancer. For example, vasoactive intestinal peptide (VIP) receptor is known to be overexpressed in breast cancer and lung cancer, and VIP antagonists are known to inhibit cancer growth. Thus, a peptide of the invention is probably effective in inhibiting such cancers;
- antagonists of chemokine receptors as anti-inflammatory and anti asthma drugs;
- tissue rejection;
- neuropeptide Y receptor antagonists as anti-obesity drugs;
- dopamine receptor D4 antagonists as drugs for treatment of depression, attention deficit hyperactivity disorder and schizophrenia;
- antagonists of Corticotropin-Releasing Factor Receptor for the treatment of depression and anxiety related disorders;
- angiotensin receptor antagonists as a mean of blood pressure control;
- antagonists of gastrin-releasing peptide receptor, somatostatin and gastrin receptors as anti-neoplastic agents that slow down growth of endocrine tumors;
- antagonists of opiod receptors as pain killers.

### a. Pharmaceutical compositions

The compositions for administration may be in the form of a solution, suspension, tablets, pill, capsule, powder, gel, cream, lotion, ointment, aerosol or the like. In a preferred embodiment, the compositions for administration comprise a

solution of the GPCR transmembrane peptides dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. In certain embodiments, the GPCR transmembrane peptides are provided in powder form.

The GPCR transmembrane peptides and analogs may be combined with conventional excipient, such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium, carbonate, and the like. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of the GPCR in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

**b. Administration and dosage**

The pharmaceutical composition or medium that comprises a GPCR transmembrane peptide is administered orally, parenterally, enterically, gastrically, topically, subcutaneously, rectally, locally or systemically. For example, the compounds can be injected into the bloodstream using a cannula or catheter; the vein or artery is selected to maximize delivery of cells to the affected tissue(s). Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as *Remington's Pharmaceutical Science*, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980). It is recognized that the GPCR transmembrane peptides polypeptides and related compounds described above, when administered orally, must be protected from digestion. This is typically accomplished either by complexing the protein with a composition to render it resistant to acidic and enzymatic hydrolysis or by packaging the protein in an appropriately resistant carrier such as a liposome. Means of protecting proteins from digestion are well known in the art.

In therapeutic applications, compositions are administered to a patient suffering from a disease or condition that in an amount sufficient to cure or at least partially arrest symptoms of the disease or conditions and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose."

5 Amounts effective for this use will depend upon the severity of the condition to be treated and the general state of the patient's health.

Generally, the dosage to be administered is the amount necessary to modulate a GPCR biological activity. It is understood that the dosage of a GPCR polypeptide of the present invention will be dependent upon the age, sex, health,

10 and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The ranges of effective doses provided herein are not intended to limit the inventors and represent preferred dose ranges. The most preferred dosage will be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. It is

15 contemplated that the compounds will be administered under the guidance of a physician, who will determine the exact dosages, monitor the progress of the treatment, and determine whether a given administration is successful and sufficient, or whether subsequent administrations are needed.

The concentration of compounds to be administered at a given time

20 and to a given patient will vary from 0.1  $\mu\text{g}$ -100 mg and preferably 0.1-10 mg per day per patient. The dosage and mode of administration may be chosen to achieve and optionally maintain a local concentration in fluids that contact the target cells of about 0.001-50  $\mu\text{g}/\text{ml}$ , preferably 0.1-10  $\mu\text{g}/\text{ml}$ . Dosages from 0.1 up to about 100 mg per patient per day may be used, particularly when the drug is administered to a

25 secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ. Substantially higher dosages are possible in topical administration.

Single or multiple administrations of the compositions may be necessary depending on the dosage and frequency as required and tolerated by the

30 patient. In any event, the composition should provide a sufficient quantity of the peptides of this invention to effectively treat the patient.

### c. Gene therapy

The present invention provides packageable GPCR transmembrane peptide-encoding nucleic acids for the transformation of cells *in vitro* and *in vivo*. These packageable nucleic acids can be inserted into any of a number of well known vectors for the transfection and transformation of target cells and organisms.

The nucleic acids are transfected into cells, *ex vivo* or *in vivo*, through the interaction of the vector and the target cell. The GPCR transmembrane peptide-encoding nucleic acid, under the control of a promoter, then expresses the GPCR transmembrane peptide, thereby modulating the biological activity of a target GPCR.

Such gene therapy procedures have been used to correct acquired and inherited genetic defects, cancer, and viral infection in a number of contexts. The ability to express artificial genes in humans facilitates the prevention and/or cure of many important human diseases, including many diseases which are not amenable to treatment by other therapies. As an example, *in vivo* expression of cholesterol-regulating genes, genes which selectively block the replication of HIV, and tumor-suppressing genes in human patients dramatically improves the treatment of heart disease, AIDS, and cancer, respectively. For a review of gene therapy procedures, see Anderson, *Science* (1992) 256:808-813; Nabel and Felgner (1993) *TIBTECH* 11: 211-217; Mitani and Caskey (1993) *TIBTECH* 11: 162-166; Mulligan (1993) *Science* 926-932; Dillon (1993) *TIBTECH* 11: 167-175; Miller (1992) *Nature* 357: 455-460; Van Brunt (1988) *Biotechnology* 6(10): 1149-1154; Vigne (1995) *Restorative Neurology and Neuroscience* 8: 35-36; Kremer and Perricaudet (1995) *British Medical Bulletin* 51(1) 31-44; Haddada et al. (1995) in *CURRENT TOPICS IN MICROBIOLOGY AND IMMUNOLOGY* Doerfler and Böhm (eds) Springer-Verlag, Heidelberg Germany; and Yu et al., *GENE THERAPY* (1994) 1:13-26.

Delivery of the gene or genetic material into the cell is the first critical step in gene therapy treatment of disease. A large number of delivery methods are well known to those of skill in the art. Such methods include, for example liposome-based gene delivery (Debs and Zhu (1993) WO 93/24640; Mannino and Gould-Fogerite (1988) *BioTechniques* 6(7): 682-691; Rose U.S. Pat No. 5,279,833; Brigham (1991) WO 91/06309; and Felgner et al. (1987) *Proc. Natl. Acad. Sci. USA* 84: 7413-7414), and replication-defective retroviral vectors harboring a therapeutic polynucleotide sequence as part of the retroviral genome (see, e.g., Miller et al.

(1990) *Mol. Cell. Biol.* 10:4239 (1990); Kolberg (1992) *J. NIH Res.* 4:43, and Cornetta et al. *Hum. Gene Ther.* 2:215 (1991)). Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SIV), human immuno deficiency virus (HIV), and combinations thereof. See, e.g., Buchscher et al. (1992) *J. Virol.* 66(5) 2731-2739; Johann et al. (1992) *J. Virol.* 66 (5):1635-1640 (1992); Sommerfelt et al., (1990) *Virology* 176:58- 59; Wilson et al. (1989) *J. Virol.* 63:2374-2378; Miller et al., *J. Virol.* 65:2220-2224 (1991); Wong-Staal et al., PCT/US94/05700, and Rosenberg and Fauci (1993) in *Fundamental Immunology, Third Edition* Paul (ed) Raven Press, Ltd., New York and the references therein, and Y et al., *GENE THERAPY* (1994), *supra*.

AAV-based vectors are also used to transduce cells with target nucleic acids, e.g., in the *in vitro* production of nucleic acids and peptides, and in *in vivo* and *ex vivo* gene therapy procedures. See, West et al. (1987) *Virology* 160:38- 47; Carter et al. (1989) U.S. Patent No. 4,797,368; Carter et al. WO 93/24641 (1993); Kotin (1994) *Human Gene Therapy* 5:793- 801; Muzyczka (1994) *J. Clin. Invest.* 94:1351 and Samulski (*supra*) for an overview of AAV vectors. Construction of recombinant AAV vectors are described in a number of publications, including Lebkowski, U.S. Pat. No. 5,173,414; Tratschin et al. (1985) *Mol. Cell. Biol.* 5(11):3251-3260; Tratschin, et al. (1984) *Mol. Cell. Biol.*, 4:2072-2081; Hermonat and Muzyczka (1984) *Proc. Natl. Acad. Sci. USA*, 81:6466-6470; McLaughlin et al. (1988) and Samulski et al. (1989) *J. Virol.*, 63:03822-3828. Cell lines that can be transformed by rAAV include those described in Lebkowski et al. (1988) *Mol. Cell. Biol.*, 8:3988-3996.

#### *i. in vitro gene transfer*

It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of DNA encoding GPCR transmembrane peptides or a peptide fragment thereof. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes is made here.

There are several well-known methods of introducing nucleic acids into bacterial and animal cells, any of which may be used in the present invention. These include: calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing the DNA, treatment of the recipient cells with



liposomes containing the DNA, DEAE dextran, receptor-mediated endocytosis, electroporation, micro-injection of the DNA directly into the cells, infection with viral vectors, etc.

For *in vitro* applications, the delivery of nucleic acids can be to any cell grown in culture, whether of bacterial, plant or animal origin, vertebrate or invertebrate, and of any tissue or type. Contact between the cells and the genetically engineered nucleic acid constructs, when carried out *in vitro*, takes place in a biologically compatible medium. The concentration of nucleic acid varies widely depending on the particular application, but is generally between about 1  $\mu$  mol and about 10 mmol. Treatment of the cells with the nucleic acid is generally carried out at physiological temperatures (about 37° C) for periods of time of from about 1 to 48 hours, preferably of from about 2 to 4 hours.

In one group of embodiments, a nucleic acid is added to 60-80% confluent plated cells having a cell density of from about  $10^3$  to about  $10^5$  cells/mL, more preferably about  $2 \times 10^4$  cells/mL. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2  $\mu$ g/mL, more preferably about 0.1  $\mu$ g/mL.

#### **ii. *In vivo* gene transfer**

Alternatively, the GPCR transmembrane peptide encoding nucleic acids can also be introduced into target cells *in vivo*, using recombinant methods which are known to those of skill in the art. The insertion of genes into cells for the purpose of medicinal therapy is a rapidly growing field in medicine which has enormous clinical potential. Research in gene therapy has been on-going for several years, and has entered human clinical trials. Zhu, et al., *Science*, 261:209-211 (1993), incorporated herein by reference, describes the intravenous delivery of cytomegalovirus (CMV)-chloramphenicol acetyltransferase (CAT) expression plasmid using DOTMA-DOPE complexes. Hyde, et al., *Nature*, 362:250-256 (1993), incorporated herein by reference, describes the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to epithelia of the airway and to alveoli in the lung of mice, using liposomes. Brigham, et al., *Am. J. Med. Sci.*, 298:278-281 (1989), incorporated herein by reference, describes the *in vivo* transfection of lungs of mice with a functioning prokaryotic gene encoding the intracellular enzyme chloramphenicol acetyltransferase (CAT).

Formulations suitable for administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can  
5 include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations of packaged nucleic acid can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials. Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

10 For *in vivo* administration, pharmaceutical compositions that comprise GPCR transmembrane peptide-encoding nucleic acids are preferably administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. More preferably, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection. For example, see Stadler, et  
15 *al.*, U.S. Patent No. 5,286,634, which is incorporated herein by reference. Intracellular nucleic acid delivery has also been discussed in Straubinger, et *al.*, *Methods in Enzymology*, Academic Press, New York. 101:512-527 (1983); Mannino, et *al.*, *Biotechniques*, 6:682-690 (1988); Nicolau, et *al.*, *Crit. Rev. Ther. Drug Carrier Syst.*, 6:239-271 (1989), and Behr, *Acc. Chem. Res.*, 26:274-278 (1993). Still other  
20 methods of administering therapeutics are described in, for example, Rahman et *al.*, U.S. Patent No. 3,993,754; Sears, U.S. Patent No. 4,145,410; Papahadjopoulos et *al.*, U.S. Patent No. 4,235,871; Schneider, U.S. Patent No. 4,224,179; Lenk et *al.*, U.S. Patent No. 4,522,803; and Fountain et *al.*, U.S. Patent No. 4,588,578.

In preferred embodiments, the pharmaceutical preparations may be  
25 contacted with the target tissue by direct application of the preparation to the tissue. The application may be made by topical, "open" or "closed" procedures. By "topical", it is meant the direct application of the pharmaceutical preparation to a tissue exposed to the environment, such as the skin, oropharynx, external auditory canal, and the like. "Open" procedures are those procedures which include incising  
30 the skin of a patient and directly visualizing the underlying tissue to which the pharmaceutical preparations are applied. This is generally accomplished by a surgical procedure, such as a thoracotomy to access the lungs, abdominal laparotomy to access abdominal viscera, or other direct surgical approach to the target tissue.

"Closed" procedures are invasive procedures in which the internal target tissues are not directly visualized, but accessed via inserting instruments through small wounds in the skin. For example, the preparations may be administered to the peritoneum by needle lavage. Likewise, the preparations may be administered through  
5 endoscopic devices.

The nucleic acid can also be administered in an aerosol inhaled into the lungs (see, Brigham, et al., *Am. J. Sci.*, 298(4):278-281 (1989)) or by direct injection at the site of disease (Culver, *Human Gene Therapy*, MaryAnn Liebert, Inc., Publishers, New York. pp.70-71 (1994)).

10 Effective doses of the compositions of the present invention will vary depending upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages will need to be titrated to optimize safety and efficacy. In determining the effective amount of the vector to be administered, the physician evaluates the  
15 particular nucleic acid used, the disease state being diagnosed; the age, weight, and condition of the patient, circulating plasma levels, vector toxicities, progression of the disease, and the production of anti-vector antibodies. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular vector. Doses ranging from about 10  
20 ng to 1 g, 100 ng to 100 mg, 1  $\mu$ g to 10 mg, or 30-300  $\mu$ g DNA per patient are typical. Doses generally range between about 0.01 and about 50 mg per kilogram of body weight; preferably between about 0.1 and about 5 mg/kg of body weight or about  $10^8$ - $10^{10}$  or  $10^{12}$  particles per injection. In general, the dose equivalent of a naked nucleic acid from a vector is from about 1  $\mu$ g to 100  $\mu$ g for a typical 70  
25 kilogram patient, and doses of vectors which include a retroviral particle are calculated to yield an equivalent amount of inhibitor nucleic acid.

Prior to infusion, blood samples are obtained and saved for analysis. Between  $10^8$  and  $1 \times 10^{12}$  vectors are infused intravenously over 60-200 minutes. Vital signs and oxygen saturation by pulse oximetry are closely monitored. Blood  
30 samples are obtained 5 minutes and 1 hour following infusion and saved for subsequent analysis. At the physician's discretion, reinfusion is repeated every 2 to 3 months for a total of 4 to 6 treatments in a one year period. After the first treatment, infusions can be performed on an outpatient basis at the discretion of the clinician. If

the reinfusion is given as an outpatient, the participant is monitored for at least 4, and preferably 8 hours following the therapy.

If a patient undergoing infusion of a vector or transduced cell develops fevers, chills, or muscle aches, he/she receives the appropriate dose of aspirin, ibuprofen or acetaminophen. Patients who experience reactions to the infusion such as fever, muscle aches, and chills are premedicated 30 minutes prior to the future infusions with either aspirin, acetaminophen, or diphenhydramine. Meperidine is used for more severe chills and muscle aches that do not quickly respond to antipyretics and antihistamines. Vector infusion is slowed or discontinued depending upon the severity of the reaction.

*In vivo* gene transfer may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as humans, non-human primates, dogs, cats, cattle, horses, sheep, and the like.

## EXAMPLES

The following examples are simply embodiments of the invention and are not intended to limit the invention. A person of ordinary skill in the art can modify and/or adapt the invention for various applications without undue experimentation, without departing from the generic concept of the present invention. Therefore, such adaptations and modifications are within the scope and range of the present invention.

### Example 1

Example 1 illustrates that peptides derived from transmembrane regions of CXCR4 inhibit CXCR4-mediated calcium fluxes.

Peptides having the selected sequences were synthesized by a flow-through solid phase peptide synthesis on 432A Applied Biosystems Peptide Synthesizer utilizing Fmoc amino acid derivatives. To overcome the aggregation that frequently occurs during the synthesis of hydrophobic peptides and leads to the blockage of the growing peptide chain, FmocHmb derivatives of Ala, Val and Leu were introduced into the difficult sequences. Charged residues were added to the peptide termini to assure a proper orientation of the peptides during penetration into

the cellular membrane, and to improve the solubility of the highly hydrophobic peptides.

The purity of the peptides was assessed by reverse phase HPLC and the structures were confirmed by matrix-assisted laser-desorption time-of-flight (MALDI-TOF) mass spectrometry (Tarasova et al. (1998), *Ad. Exp. Med. Biol.*, Plenum Press, NY, pp. 201-206.)

Peptides used in this example are listed in Tables 1, 2, and 3.

The effect of the peptides on CXCR4-mediated calcium fluxes in HeLa cells that naturally express the CXCR4 receptor and U87 cells stably expressing the CXCR4 receptor was tested as follows. Cells grown on Nunc cover glass chamber slides were incubated with 1 micromolar Fura-2/AM for 20 min in a CO<sub>2</sub>-incubator, rinsed with PBS and mounted on the stage of a Zeiss Axiovert inverted microscope. [Ca<sup>2+</sup>]<sub>i</sub> measurements were performed using an Attofluor digital imaging system (Atto Instruments, Rockville, MD). Fluorescence of Fura was excited at alternating wavelength of 340 and 380 nm. Fluorescence was monitored by an intensified CCD camera using a 505 cut-off filter. Calibrations of [Ca<sup>2+</sup>]<sub>i</sub> signals were performed using Ca<sup>2+</sup> standards containing 1 micromolar Fura. CXCR4 antagonists were tested on HeLa cells and U87 cells. Stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) was used as a specific CXCR4 agonist.

CCR5 antagonists, which were used in selectivity studies as described below, were tested on HEK (human kidney carcinoma) cells stably expressing the CCR5 receptor and RANTES was used as an agonist. The antagonist activity of the peptides was evaluated by measuring the inhibition of agonist-evoked intracellular Ca<sup>2+</sup><sub>i</sub> release. These measurements were carried out in Fura-2/AM-treated cells, utilizing an Attofluor digital imaging system as described above. The agonist was SDF-1 $\alpha$ .

In the preliminary screen, peptides corresponding to the second and sixth transmembrane domains were found to abolish SDF-1 $\alpha$  - induced signaling through CXCR4 receptor (Table 1). Further optimization and structure-activity studies allowed to obtain antagonists derived from all but the third and fifth transmembrane domains (Table 2).

**Table 1.** Activity of synthetic peptides corresponding to predicted transmembrane domains of CXCR4 in inhibition of SDF-1 $\alpha$ -induced intracellular calcium release.

5	<p>Peptide</p>	<p>Concentration, required for complete inhibition of [Ca<sup>2+</sup>]<sub>i</sub> release</p>
	<p>F-1-5: DDIFLPTIYSIIFLTGIV-HN<sub>2</sub></p>	<p>&gt; 30 <math>\mu</math>M</p>
10	<p>F-2-1: LLFVITLPFWAVDAVANWYFGN-OH</p>	<p>5 <math>\mu</math>M</p>
	<p>F-3-1: KAVHVIYTVNLYSSVLILAFISL-NH<sub>2</sub></p>	<p>&gt; 50 <math>\mu</math>M</p>
15	<p>F-4-1: KVYVGWVWIPALLLTIPDFIF-OH</p>	<p>&gt; 50 <math>\mu</math>M</p>
	<p>F-5-1: HIMVGLILPGIVILSCYCIII-NH<sub>2</sub></p>	<p>&gt; 50 <math>\mu</math>M</p>
20	<p>F-6-1: VILILAFFACWLPYYIGISID-OH</p>	<p>10 <math>\mu</math>M</p>
25	<p>F-7-1: ALAFFHCCLNPILYAFLGAK-NH<sub>2</sub></p>	<p>&gt; 100 <math>\mu</math>M</p>

**Table 2.** Biological activity of CXCR4 antagonists derived from different transmembrane domains. Anti signaling activity was determined in inhibition of SDF-1 $\alpha$ -induced intracellular calcium release. Anti-HIV-1 activity was assessed in cytoprotection assay utilizing CEM-SS cells infected with HIV-1<sub>RF</sub>.

Peptide	Concentration, required for inhibition of signal transduction ( $\mu$ M)	EC <sub>50</sub> in anti-HIV-1 assay ( $\mu$ M)
F-2-2 LLFVITLPFWAVDAVANWYFGNDD	0.2	2.27
F-4-2 VYGVWIPALLLTIPDFIFANDD-OH	5	0.3
F-6-1 VILILAFFACWLPPYYIGISID-OH	10	> 50
F-7-3 DDEALAFFHCCLNPILYAFL-NH <sub>2</sub>	25	3.27
F-6-1 + F-7-3	1	No data

To further understand the structural requirements for a successful antagonist, structure-activity studies were conducted on the peptides derived from the second transmembrane domain of CXCR4 (Table 3). The most potent antagonist, a 24 amino acid residue peptide F-2-2, completely blocked signal transduction at 0.2 micromolar. Addition of negatively charged residues to the termini appeared to be important for the activity. Elimination of the added negative charges provided by two C-terminal Asp residues (F-2-1) decreased antagonist potency more than ten-fold. Consistent with those findings, the substitution of negatively charged aspartate residues with positively charged lysines (F-2-3) resulted in 100-fold decrease in antagonist activity. Deletion of five residues preceding the C-terminal aspartates (F-2-4) reduced the potency 20 -fold. Truncation of the transmembrane portion by three N-terminal residues Leu-Leu-Phe rendered the peptide inactive.



**Table 3.** Structure-activity relationships in peptides derived from the second transmembrane domain of CXCR4 :

...HLSVADLLFVITLPFWAVDAVANWYFGNFLCK... (predicted intramembrane portion is underlined)

5	Peptide	Concentration, required for complete inhibition of [Ca <sup>2+</sup> ] release
	F-2-1: LLFVITLPFWAVDAVANWYFGN-OH	5 $\mu$ M
10	F-2-2: LLFVITLPFWAVDAVANWYFGN <u>DD</u> -OH	0.2 $\mu$ M
15	F-2-8: LLFVITLPFWAVDAVANWYFGN <u>KK</u> -OH	20 $\mu$ M
	F-2-4: VITLPFWAVDAVANWYFGN <u>KK</u> -OH	> 50 $\mu$ M
20	F-2-5: LLFVITLPFWAVDAVAN <u>DD</u> -OH	10 $\mu$ M
	AcF-2-5: AcLLFVITLPFWAVDAVAN <u>DD</u> -OH	10 $\mu$ M
25	F-2-6: LSVADLLFVITLPFWAVDAVAN <u>DD</u> -OH	20 $\mu$ M
30	Rhod - F-2: AcLLFVITLPFWAVDAVANWYFGNDDK(Rhod)D-OH	8 $\mu$ M

Similar results were also observed for peptides derived from additional transmembrane regions. For example, in the case of peptides corresponding to the fourth transmembrane domain, positioning of the charged residue at the intracellular end of the peptide (F-4-1, Table 1) instead of the extracellular end (F-4-2, Table 2) abolished the antagonist activity. Further, substitution of extracellular aspartates with lysines also abolished the antagonist activity (data not shown).

The specificity of the transmembrane domain interaction was demonstrated by the fact that all peptides derived from CXCR4 showed selectivity for that receptor and had no influence on signaling of the other chemokine receptor involved in HIV-1 entry, CCR5. Similarly, a peptide derived from the second transmembrane domain of CCR5, LFFLLTVPFWAHYAAAQWDFGDD, completely abolished agonist induced signaling of the receptor in U87 cells at 500 nM concentrations, but had no effect on signaling of CXCR4.

It was further noted that an equimolar mixture of two peptides, F-6-1 and F-7-3, was an order of magnitude more potent than the most active of the two peptides. This synergistic effect produced by the derivatives of the sixth and seventh transmembrane regions may be a general phenomenon. Thus, pairs of TM analogs in optimized combinations may act as very potent antagonists.

#### Example 2

Example 2 illustrates that synthetic peptides corresponding to transmembrane domains of CXCR4 inhibit CXCR4-mediated HIV infection.

CCR5 and CXCR4 are believed to be the main co-receptors for HIV-1 cell entry (Broder et al. (1997), *J. Leukoc. Biol.* 62:2029; Doranz et al. (1997) *Immunol. Res.* 16: 1528; Premack and Schall (1996), *Nat. Med.* 2:1174-1178.), although other chemokine receptors appear to mediate infection as well (Michael et al. (1997) *Nat. Med.* 3(10):11602).

The ability of synthetic CXCR4-derived peptides of Table 1 to inhibit HIV-1 infection of CEM-SS cells was tested using an LAV strain of the virus that is known to utilize CXCR4 as a co-receptor. Anti-HIV-1 assay. Buckheit et al. (1993) *Antiviral Research* 21: 247. The CEM-SS cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum. The cells were placed in each well of a 96-well microtiter plate to a density of  $5 \times 10^3$  cells per well. The cells were infected with HIV-1 virus at a multiplicity of infection (MOI) previously determined to produce maximal level of viral production at 6 days post infection (MOI of 0.01).

Serial half-log dilutions of test compound were added to appropriate wells in triplicate to evaluate their ability to inhibit HIV-1 infection. AZT was used in parallel as a positive control. Following 6 days of incubation at 37° C, the presence and relative abundance of viral p24 protein was determined by ELISA in cell-free supernatants derived from each well of the microtiter plate. The p24 ELISA kit was purchased from the AIDS Vaccine Program, NCI, FCRDC (Frederick, MD) and the assay was performed according to the manufacturer's instructions.

Most of the peptides in Table 1 showed some antiviral activity (data not shown). However, the peptides corresponding to the second and sixth transmembrane domains were the most potent in inhibition of HIV entry. The F-2-2 compound completely inhibited infection at a 5 micromolar concentration (Fig. 1).

Peptides corresponding to transmembrane domains of the cholecystokinin type A receptor (CCKAR) were used as negative controls and did not effect CXCR4 function, thereby confirming the specificity of the effect.

The peptides showed no cell toxicity in concentrations up to 100 micromolar (higher concentrations could not be tested because of solubility problems).

The ability of synthetic peptides to inhibit HIV-1 infection was additionally tested by cytoprotection assay using the highly cytopathic HIV-1 strain RF (Rice, et al. (1995) *Adv. Pharmacol.* 33:389,) (Table 2). The most potent peptide, F-4-2, completely inhibited infection at 1 micromolar concentration (Fig. 2). The peptides used as negative controls, which correspond to transmembrane domains of the cholecystokinin receptor type A, did not effect chemokine receptors functions.

The above results generally demonstrate the ability of externally added molecules to compete for interaction between transmembrane domains of GPCRs and thereby to disrupt receptor function (Figure 3). In addition, it is important to note that the peptides of the invention inhibit HIV infection by targeting a cellular molecule and function rather than a viral molecule and function. Viral proteins have a relatively high mutation rate, which often allows viruses to become resistant to a given treatment. Because cellular proteins mutate at a far slower rate, the probability that a virus will be able to develop a resistance is greatly reduced.

### Example 3

Example 3 shows that the peptides of the invention partition to the plasma membrane and other membrane compartments.

A fluorescent derivative of CXCR4 TM2, rhodamine-F-2, was synthesized by solid-phase synthesis on 432A Applied Biosystems Peptide Synthesizer utilizing Fmoc amino acid derivatives. Rhodamine B (Fluka) was loaded onto the amino acid column of the instrument. The purity of the peptide was assessed by reverse phase HPLC and the structures were confirmed by matrix-assisted laser desorption mass spectrometry.

A chimeric protein consisting of the CXCR4 and the green fluorescent protein (GFP) was used for studying receptor localization, internalization, and recycling in live cells in real time. This construct was made and stably expressed in HeLa cells as described in Tarasova et al. (1997), *J. Biol. Chem.* 272: 14817-14824. Fusion of the C terminus of the CXCR4 to the N terminus of the GFP did not appear to alter receptor ligand binding affinity, signal transduction, or the pattern of receptor surface expression and distribution.

Transfected CXCR4-GFP-expressing HeLa cells were grown in coated 50 mm cover glass bottom dishes (MatTek, MA) in medium without phenol red. The cells were then exposed to 1 micromolar peptide in DMEM medium for 30 min in a CO<sub>2</sub>-incubator. The distribution of fluorescent label was determined by confocal laser scanning microscopy on a Zeiss inverted LSM 410 laser scanning confocal microscope. Fluorescence of GFP was excited using a 488 nm argon/krypton laser; emitted fluorescence was detected with 515-540 nm bandpass filter. For rhodamine red a 568 nm helium/neon laser was used for excitation and fluorescence was detected with a 590-640 nm bandpass filter.

The results demonstrated that the rhodaminated peptide co-localized with the CXCR4-GFP and was present at the cellular membrane within minutes after application and saturated endosomes and the endoplasmic reticulum after 15 minutes of incubation. This confirmed the ability of the peptides to concentrate in the cellular membranes and suggested that the peptides interacted with receptor molecules.

#### Example 4

Example 4 shows that peptides corresponding to transmembrane domains of the cholecystokinin type A receptor (CCKAR) inhibits agonist-evoked intracellular calcium release with a potency similar to CXCR4 compounds.

To further illustrate the present invention, we have synthesized peptides derived from the transmembrane domains of the rat cholecystokinin receptor type A (CCKAR). Although CCKAR belongs to the same rhodopsin family of GPCRs as CXCR4, its sequence is only 15% identical to that of CXCR4, when aligned using the Dialign 2

program (Morgenstern, et al., (1996) *Proc. Natl. Acad. Sci. USA* 93:12098) and the degree of identify in transmembrane regions is only 27%.

The activity of peptides from the CCKAR transmembrane domain were tested in transfected CHO cells that stably express rat CCKAR (Tarasova et al. (1997), *J. Biol. Chem.* 272: 14817-14824). Sulfated cholecystokinin octapeptide was the CCKAR agonist. Determination of intracellular calcium release was performed as described in example 1 above. The results are shown in Table 4.

None of CCKAR-derived peptides served as antagonists of chemokine receptors and none could inhibit HIV-1 infection. The activity of the antagonists in inhibiting signaling through the receptor was compared to the ability to prevent agonist binding (Table 4). Inhibition of signaling was assessed in CCK-8 evoked intracellular calcium release in FURA-2/AM treated CHO cells stably transfected with rat CCKAR (Tarasova, et al. (1997) *J. Biol. Chem.* 272:14817). Inhibition of ligand binding was measured with the use of a fluorescent agonist, rhodamine green CCK-8 (RG-CCK-8) and quantitative confocal laser scanning microscopy (Tarasova, et al. (1997) *J. Biol. Chem.* 272:14817). Peptides derived from the first, second, and sixth transmembrane domains inhibited CCK-induced signaling through the CCKAR receptor. Peptides derived from the first and the second transmembrane domains, CCKAR-1-1 and CCKAR-2-1, had comparable potencies with respect to the inhibition of ligand signaling and binding. A peptide derived from the sixth domain, CCKAR-6-1, was active in inhibition of signaling, but had very low activity in inhibition of RG-CCK-8 binding.

Table 4. The activity of CCKAR-derived TM peptides in inhibition of CCK-8 -induced intracellular calcium release and RG-CCK-8 binding.

Peptide	Concentration, required for inhibition of signaling	IC <sub>50</sub> in inhibition of RG-CCK-8 binding
CCKAR-1-6: DDEWQSALQILLYSIIFLLSVGNTLVITV	50 $\mu$ M	20 $\mu$ M
CCKAR-2-1: FLLSLAVSDLMLCLFCMPFNLP	2 $\mu$ M	0.5 $\mu$ M
CCKAR-4-2 (#71) VIAATWCLSFTIMTPYPIYSNLVPFTDD	> 50 $\mu$ M	> 50 $\mu$ M
CCKAR-5-3 (#45) DDQTFLLLILFLLPGIVMVVAYGL	> 50 $\mu$ M	> 50 $\mu$ M
CCKAR-6-4 (#77) IVVLFFLCWMPIFSANAWRAYDTVDD	5 $\mu$ M	> 50 $\mu$ M

## CLAIMS

## WHAT IS CLAIMED IS:

- 5                   1. An isolated G protein-coupled receptor (GPCR)-modulating molecule comprising a peptide or peptidomimetic that is a structural analog of a portion of a transmembrane domain of a GPCR, wherein  
                       said molecule has a first end and a second end and said molecule has at said first end a negatively charged group and at said second end a neutral charge under  
 10   physiological conditions;  
                       said molecule spontaneously inserts into a membrane in the same orientation as the transmembrane GPCR domain from which it is derived; and  
                       said molecule modulates a biological property or activity of said GPCR by disrupting the structure or assembly of said GPCR.
- 15                   2. A molecule of Claim 1, wherein the negatively charged group at the first end of the molecule is provided by one to three acid residues which are acidic under physiological conditions.
- 20                   3. A molecule of Claim 1, wherein the negatively charged group at the first end of the molecule is provided by a carboxyl, phosphate, borate, sulfonate or sulfate group.
- 25                   4. An isolated G protein-coupled receptor (GPCR)-modulating molecule comprising a peptide or peptidomimetic that is a structural analog of a portion of a transmembrane domain of CXCR4,  
                       wherein said portion of said transmembrane domain has a sequence selected from the group of sequences consisting of:  
                       LLFVITLPFWAVDAVANWYFGNDD,  
 30   LLFVITLPFWAVDAVANDD-OH,  
                       VYVGWIPALLLTIPDFIFANDD-OH,  
                       VILILAFFACWLPYYIGISID-OH,  
                       DDEALAFFHCCLNPILYAFL-NH<sub>2</sub>,

and

DDSITEALAFFHCCLNPILYAFL-NH<sub>2</sub>,

and wherein said molecule modulates a biological activity of said CXCR4.

5                    5. A G protein-coupled receptor (GPCR)-modulating molecule of Claim 4, wherein the CXCR4 activity modulated by said peptide is inhibition of CXCR4-mediated intracellular Ca<sup>2+</sup> release.

6. A G protein-coupled receptor (GPCR)-modulating molecule of Claim 4,  
10 wherein the CXCR4 activity modulated by said peptide is inhibition of CXCR4-mediated HIV infection.

7. A method of modulating the biological activity of a target GPCR by contacting a cell that expresses said GPCR with a molecule of Claim 1.

15

8. A method of claim 7, wherein the concentration of the molecule is about 0.01 to about 100 micromolar.

9. A method of claim 7, wherein the molecule inhibits a biological property  
20 or activity of GPCR.

10. A method of claim 7, wherein the molecule that inhibits a biological property or activity of GPCR comprises a peptide or peptidomimetic that is a structural analog of a portion of a transmembrane domain of a GPCR, selected from the group  
25 consisting of:

From the GPCR CXCR4

F-2-2: LLFVITLPFWAVDAVANWYFGNDD

F-2-5: LLFVITLPFWAVDAVANDD-OH

30 F-4-2: VYVGWIPALLLTIPDFIFANDD-OH

F-6-1: VILIAFFACWLPHYIGISID-OH

F-7-3: DDEALAFFHCCLNPILYAFL-NH<sub>2</sub>

F-7-4: DDSITEALAFFHCCLNPILYAFL-NH<sub>2</sub>



From the GPCR CCR5

CCR5-TM-2-2: LFFL LTVPFWAHYAAAQWDFGDD

CCR5-TM-4-1: FGVVTSVITWVVAVFASLPGIIFTSSDD

CCR5-TM-6-1: LIFTIMIVYFLFWAPYNIVLLLNTFQED

5 CCR5-TM-7-1: DDQAMQVTETLGMTHCCINPIIYAFV

From the GPCR CCR2

CCR2-TM-2-1: IYLLNLAISDLLFLITLPLWADD-OH

CCR2-TM-2-2: LLFLITLPLWAH SAANEVWVFGNDD-OH

10 CCR2-TM-4-1: FGVVTSVITWLVAVF ASVPGIIFTDD

CCR2-TM-6-1: VIFTIMIVYFLFWTPYN IVILLNTFQED

CCR2-TM-7-1: DDATQVT ETLGMTHCCINPIIYAFV

From the GPCR CCR3

15 CCR3-TM-2-1: LLFLVTLPFW IHYVRGHNWVFGDDD

CCR3-TM-4-1: FGVITSIVTWGLAVLAALPEFI FYETED

CCR3-TM-6-1: IFVIMAVFFI FWTPYNVAILLSSYQSDD

CCR3-TM-7-1: DDLVMLVTEVIAYSHCCMNPVIYAFV

20 From the GPCR CCKAR

CCKAR-TM-1-6: DDEWQSALQILLYSIIFLLSVLGNTLVITV

CCKAR-TM-2-2: FLLSLAVSDLMLCLFCM PFNLIDD

CCKAR-TM-6-4: IVVLFFLCWMPIFSANAWRAYDTVDD.

- 25 11. A method of claim 9, wherein the inhibited biological activity is selected from the group consisting of altered ion flux or translocation, altered phosphorylation, altered protein synthesis or degradation, altered cellular morphology, altered secretion, altered production of particular components such as soluble inositol phosphates, binding of a virus and subsequent infection, tumor growth, chemotaxis,
- 30 mitogenic response, cell growth activation, secretion, muscle contraction, vasopressing and vasodepressing activity, synaptic transmission, and release of intracellular calcium.

12. A method of modulating the biological activity of a GPCR by contacting a cell that expresses said GPCR with a molecule of Claim 4.

13. A method of claim 12, wherein the concentration of the molecule is  
5 about 0.2 to about 10 micromolar.

14. A method of claim 12, wherein the target GPCR is CXCR4 and the modulated biological activity is inhibition of CXCR4-mediated HIV infection.

10 15. A method of claim 12, wherein the target GPCR is CXCR4 and the modulated biological activity is inhibition of CXCR4-mediated intracellular  $\text{Ca}^{2+}$  release.

16. A method of claim 12, wherein the target GPCR is CCR5 and the modulated biological activity is inhibition of CCR5-mediated HIV infection.

15 17. A method of claim 12, wherein the target GPCR is CCR5 and the modulated biological activity is inhibition of CCR5-mediated intracellular  $\text{Ca}^{2+}$  release.

18. A method of claim 12, wherein the target GPCR is CCKAR and the  
20 modulated biological activity is inhibition of CCKAR-mediated intracellular  $\text{Ca}^{2+}$  release.

19. A method of claim 12, wherein the target GPCR is CCKAR and the modulated biological activity is inhibition of tumor growth or progression.

25 20. A method of inhibiting HIV-1 infection, comprising contacting a cell that expresses a GPCR that binds HIV-1 with a molecule that comprises a peptide or peptidomimetic that is a structural analog of a portion of a transmembrane domain of said GPCR, wherein contacting the cell with said molecule inhibits HIV-1 infection.

30 21. A method of Claim 20, wherein the peptide or peptidomimetic is a structural analog of a portion of a transmembrane domain of CXCR4, CCR2, CCR3, or CCR5.

22. A method of Claim 21, comprising contacting a CXCR4-expressing cell with a molecule that comprises a CXCR4 transmembrane peptide having a sequence selected from the group of sequences consisting of:

LLFVITLPFWAVDAVANWYFGNDD,

- 5 LLFVITLPFWAVDAVANDD-OH,  
VYVGWIPALLLTIPDFIFANDD-OH,  
VILILAFFACWLPHYIGISID-OH,  
DDEALAFFHCCLNPILYAFL-NH<sub>2</sub>,

and

- 10 DDSITEALAFFHCCLNPILYAFL-NH<sub>2</sub>,

and wherein said molecule is effective to inhibit CXCR4-mediated infection of CXCR4-expressing cells with said HIV virus.

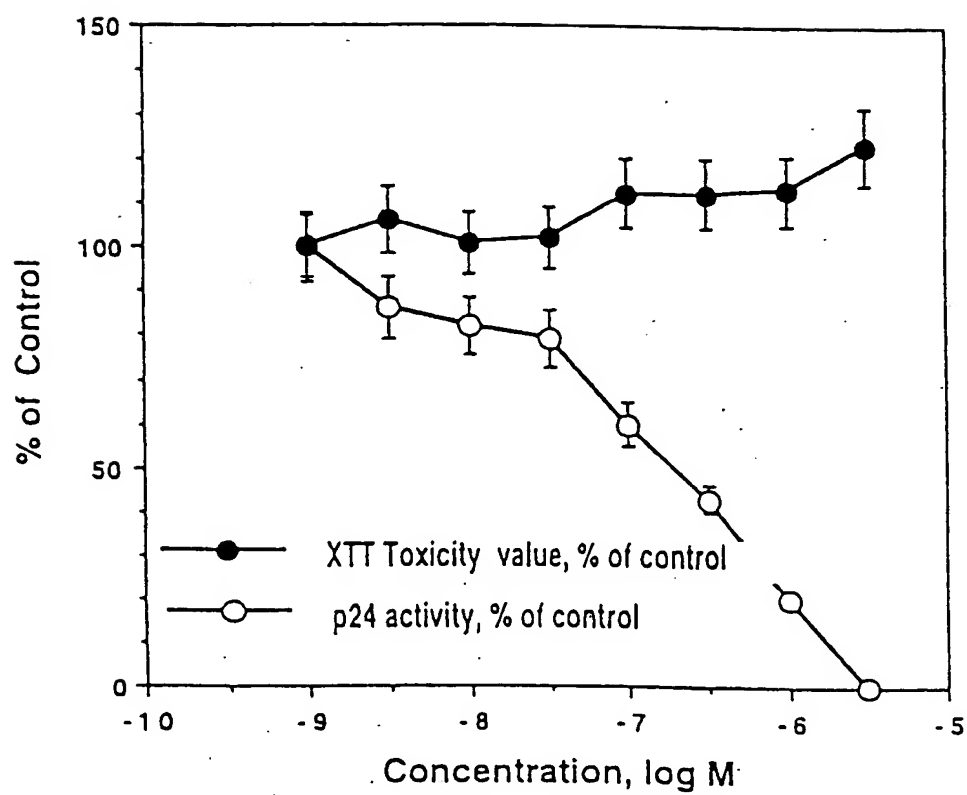


Figure 1.

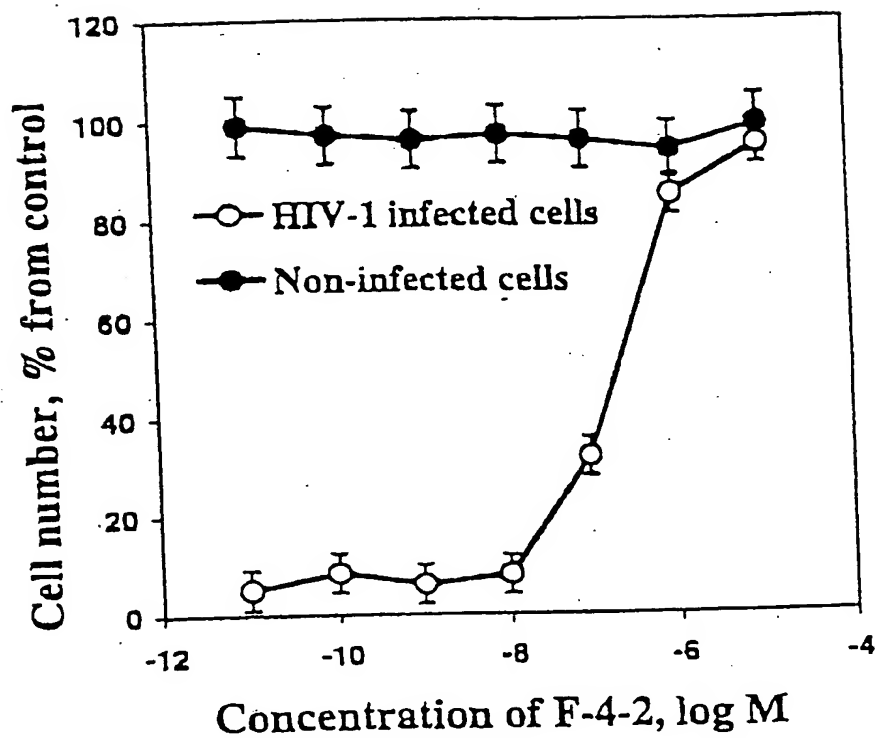


Figure 2.

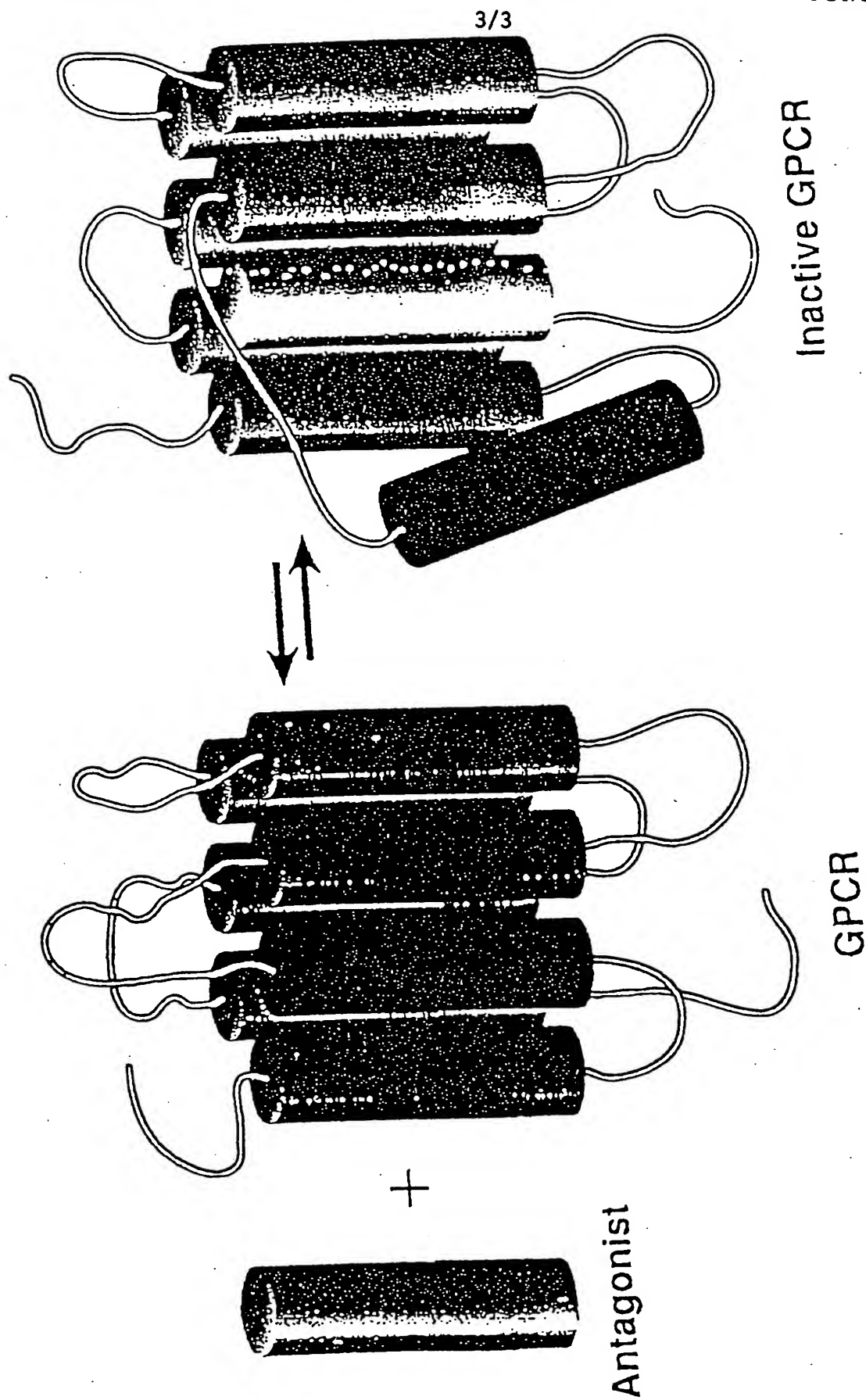


Figure 3.

# INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/US 99/04438

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07K14/715 A61K38/19

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MORO O ET AL: "Hydrophobic amino acid in the 12 loop plays a key role in receptor -G protein coupling." JOURNAL OF BIOLOGICAL CHEMISTRY, (1993 OCT 25) 268 (30) 22273-6. , XP002109857 abstract; table II ---	1-3
A	WO 97 35881 A (NG GORDON Y K ;SEEMAN PHILIP (CA); GEORGE SUSAN R (CA); DOWD BRIAN) 2 October 1997 (1997-10-02) page 32; claims 22,23,54 abstract ---	20.21
X	WO 98 00538 A (BIOSIGNAL INC ;BOUVIER MICHEL (CA); DENNIS MICHAEL (CA); UNIV MONT) 8 January 1998 (1998-01-08) page 53 abstract ---	20.21
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "3" document member of the same patent family

Date of the actual completion of the international search

21 July 1999

Date of mailing of the international search report

04/08/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040. Tx. 31 651 epo nl.  
Fax: (+31-70) 340-3016

Authorized officer

Cervigni, S

# INTERNATIONAL SEARCH REPORT

Int ernational Application No

PCT/US 99/04438

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 28258 A (NAT INST HEALTH) 7 August 1997 (1997-08-07) abstract	20,21
A	<p>---</p> <p>RAPORT C J ET AL: "Molecular cloning and functional characterization of a novel human CC chemokine receptor (CCR5) for RANTES, MIP-1beta, and MIP-1alpha" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 29, 19 July 1996 (1996-07-19), pages 17161-17166, XP002089552 ISSN: 0021-9258 figure 2</p> <p>-----</p>	



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/04438

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 7-22  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 7-22 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/04438

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9735881	A	02-10-1997	AU 2020497 A EP 0906339 A	17-10-1997 07-04-1999
WO 9800538	A	08-01-1998	AU 3705097 A CA 2205543 A CA 2259132 A EP 0910640 A	21-01-1998 01-01-1998 08-01-1998 28-04-1999
WO 9728258	A	07-08-1997	AU 1834197 A EP 0854918 A	22-08-1997 29-07-1998

Form PCT/ISA/210 (patent family annex) (July 1992)

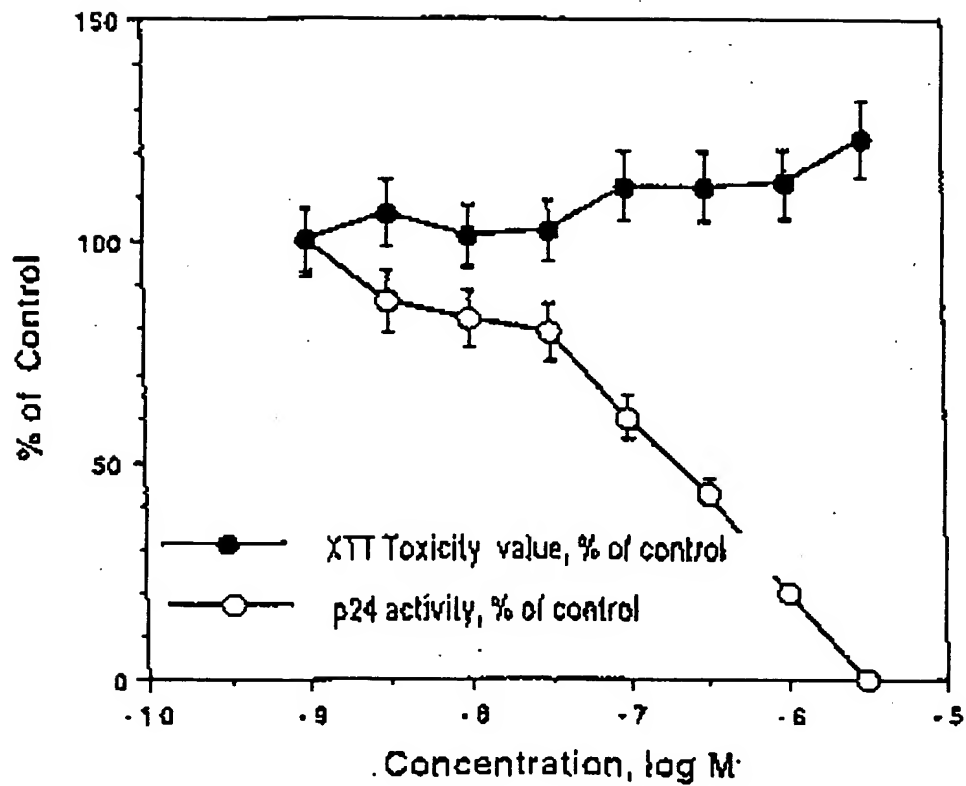


Figure 1.

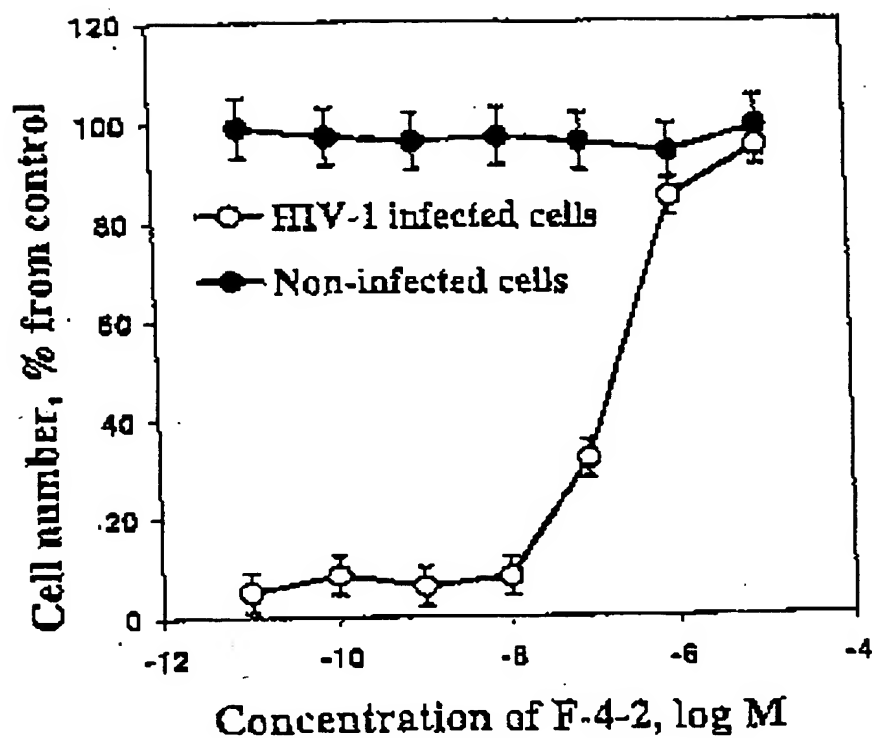


Figure 2.

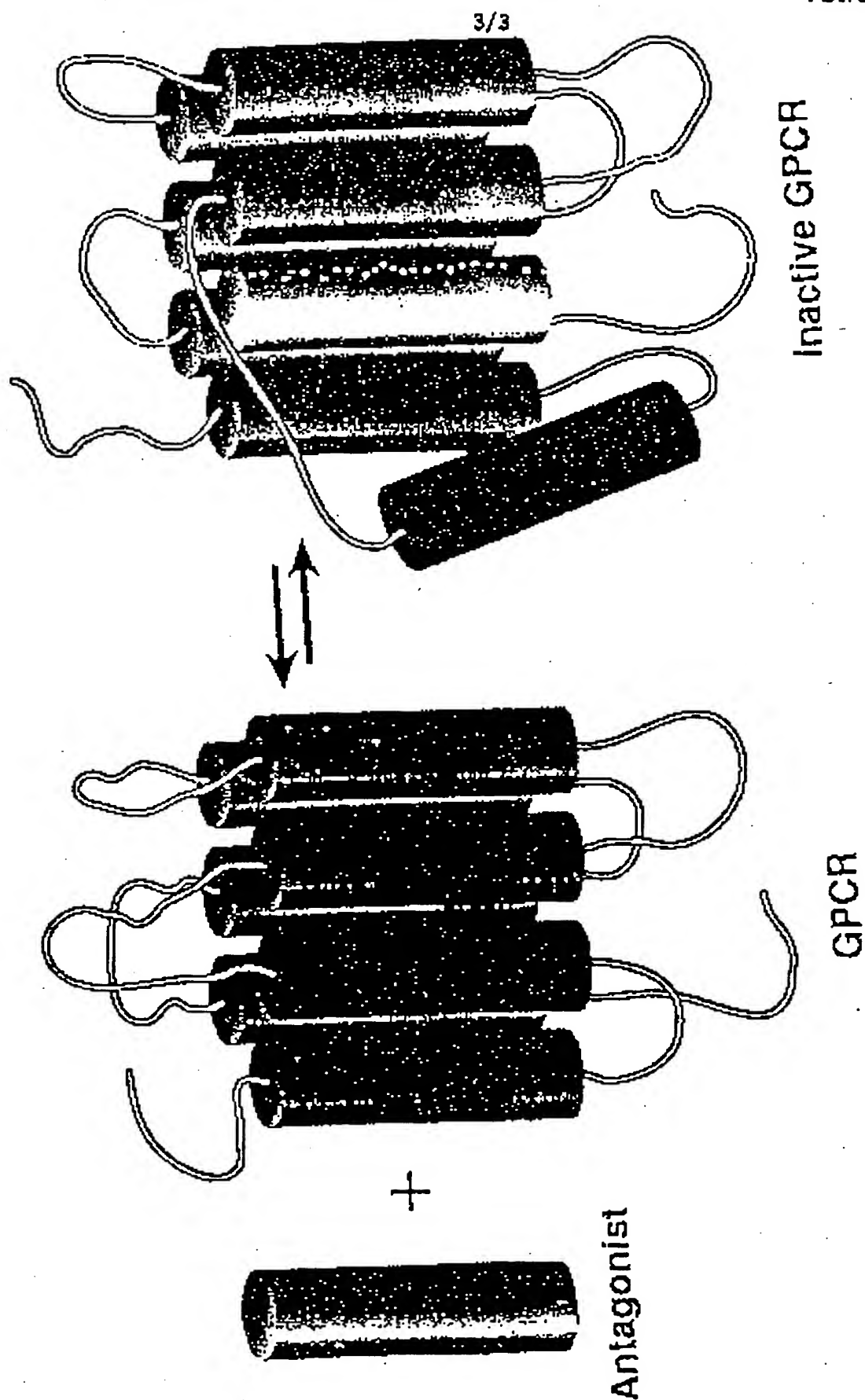


Figure 3.

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